

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
5 April 2001 (05.04.2001)

PCT

(10) International Publication Number  
**WO 01/23570 A2**

(51) International Patent Classification<sup>7</sup>: **C12N 15/12**,  
5/12, C07K 14/705, 16/28, G01N 33/50, 33/68, A61K  
38/17

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(21) International Application Number: **PCT/US00/27034**

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,  
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(22) International Filing Date:  
29 September 2000 (29.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/156,837 30 September 1999 (30.09.1999) US

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,  
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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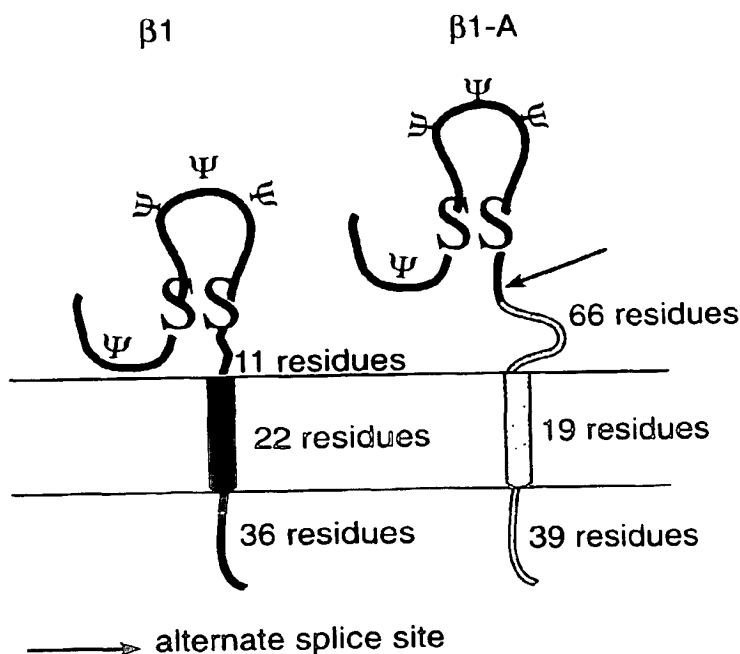
**Published:**

— Without international search report and to be republished  
upon receipt of that report.

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For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: METHODS AND COMPOSITIONS RELATING TO NOVEL SODIUM CHANNEL  $\beta$ (b)1A SUBUNITS



(57) Abstract: The present invention describes a novel subunit of voltage-gated sodium channels. This subunit is a splice variant of the  $\beta$  subunit of sodium channels that encodes a novel protein. Methods and compositions for using these nucleic acids and proteins of this subunit are described. The invention further discloses methods for the treatment of neuropathic pain.

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## METHODS AND COMPOSITIONS RELATING TO NOVEL SODIUM CHANNEL $\beta$ 1A SUBUNITS

### RELATED APPLICATIONS

5           This application claims priority to U.S. Provisional Application No. 60/156,837, filed September 30, 1999, which is herein incorporated by reference in its entirety.

### FIELD OF THE INVENTION

10           The present invention relates generally to sodium channel proteins and more particularly to the  $\beta$  subunits of voltage gated sodium channel proteins, to DNA sequences encoding these subunits, to the polypeptide products of recombinant expression of these DNA sequences, to peptides whose sequences are based on amino acid sequences deduced from these DNA sequences, and to procedures relating to the development of drugs that influence function of such proteins.

### 15           BACKGROUND OF THE INVENTION

20           Ion channels from mammalian systems are the subject of intensive scientific investigation because of the importance and variety of their biochemical functions. Ion channels are now understood to be polypeptide or protein structures with tertiary-quaternary structures forming interior pores embedded in plasma cell membranes, that control the flow of ionic currents. There are many types of ion channels which share both similarity of function and amino acid sequence, thus defining familial relationships between many of these channels. Current work shows there are ion channel families comprised of voltage-gated sodium, potassium, and calcium channels, as well as the ligand-gated acetylcholine receptors, glycine receptors, and gamma aminobutyric acid receptors.

25           Voltage-gated sodium channels have been the subject of numerous studies and much is known about these channels and their component parts.. These transmembrane proteins are responsible for the early sodium permeability increases that underlie initial depolarization of the action potential in many excitable cells such as muscle, nerve, and cardiac cells.

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More specifically, sodium channels are composed of a central pore-forming  $\alpha$  subunit (260 kDa) and two auxiliary subunits,  $\beta 1$  (36 kDa) and  $\beta 2$  (33kDa), which do not form the pore yet play critical roles in channel modulation and expression. The  $\beta 1$  subunit is of particular interest because a mutation in the  $\beta 1$  gene (*Scn1b*) has been implicated to play a role in febrile seizures and generalized epilepsy, GEFS+ (Wallace *et al.*, 1998).

The primary structure of the  $\beta 1$  subunit deduced from its cDNA sequence predicts an integral membrane glycoprotein with type I transmembrane topology as well as an extracellular immunoglobulin (Ig)-fold (Isom *et al.*, 1994; Isom and Catterall, 1996).  $\beta 1$  subunits can be classified as members of the V-set of the Ig-superfamily which includes many cell adhesion molecules.  $\beta 1$  and type IIA  $\alpha$  subunit co-expression has been well-characterized in *Xenopus* oocytes and in mammalian cells. In oocytes, co-expression of type IIA (*Scn2a*) or  $\mu 1$  (*Scn4a*)  $\alpha$  subunits with  $\beta 1$  increases the proportion of sodium channels that function in a fast gating mode, accelerates the macroscopic rates of activation and inactivation, shifts the voltage dependence of inactivation in the hyperpolarizing direction, and increases the peak current amplitude consistent with increases in channel expression (Isom *et al.*, 1992; Bennett *et al.*, 1993; Cannon *et al.*, 1993; Schreibmayer *et al.*, 1994; Wallner *et al.*, 1993). In Chinese hamster lung (CHL) cells, stable coexpression of  $\beta 1$  with  $\alpha$ IIA results in increased channel expression levels at the plasma membrane as well as moderate hyperpolarizing shifts in the voltage dependence of channel activation and inactivation (Isom *et al.*, 1995).

Northern blot analysis has shown that rat brain  $\beta 1$  mRNA is expressed only after birth in the developing brain (Patton *et al.*, 1994; Sashihara *et al.*, 1995). However, previous studies showing the developmental time course of  $\beta 1$  expression in rat forebrain showed a 26-kDa  $\beta 1$ -immunoreactive protein at embryonic day 18 (McHugh-Sutkowski and Catterall 1990). This protein was also expressed in adult adrenal gland, heart, skeletal muscle, and PC12 cells. After birth there was a dramatic decrease in the level of this protein in brain, and little if any remained by postnatal day 14. Thus, the expression time course of this immunoreactive band was complementary to that of  $\beta 1$  mRNA. Other excitable tissues express multiple size forms of immunoreactive  $\beta 1$ -like subunits. Adult rat heart and skeletal muscle membrane

preparations exhibited 38-, and 41-kDa bands on Western blots in addition to the 26-kDa band. Day 18 embryonic brain membranes also exhibited a low level of an immunoreactive peptide that migrated with an apparent molecular weight greater than 42-kDa. This protein was not detected in rat brain after birth. The 41-kDa immunoreactive band was identified as the adult rat brain isoform, and was later identified as C1Aa. $\beta$ 1 (Isom *et al.*, 1992). Because all of the immunoreactive peptides identified in the McHugh-Sutkowski and Catterall study were detected with a polyclonal antibody raised against purified  $\beta$ 1 subunits, there is no clear indication of what the identity of each of these peptides may be.

From the discussion above it is clear that voltage-gated sodium channels are of great scientific and economic interest. Further, it would appear that there is some protein in addition to the sodium channel  $\alpha$ -subunit and the  $\beta$ 1 subunit that is involved in the regulation and/or formation of functional sodium channels. The present invention is directed to identification and characterization of such a protein.

## BRIEF SUMMARY OF THE INVENTION

The present invention describes a novel splice variant of the sodium channel  $\beta$ 1 subunit and various methods and compositions for exploiting this finding. Thus, the present invention contemplates an isolated nucleic acid comprising a region, or the complement thereof, encoding a sodium channel  $\beta$ 1A subunit or an allelic variant or mutant thereof. In specific embodiments, the sodium channel  $\beta$ 1A subunit coding region encodes a primate sodium channel  $\beta$ 1A subunit. In other embodiments, the sodium channel  $\beta$ 1A is a splice variant of sodium channel  $\beta$ 1 subunit. More particularly, the splice variant results from an intron retention of the sodium channel  $\beta$ 1 subunit encoding nucleic acid. More particularly, the nucleic acid has a sequence of SEQ ID NO:1 (GenBank<sup>TM</sup> AF182949). In specific embodiments, the nucleic acid is selected from the group consisting of genomic DNA, complementary DNA and RNA. In certain embodiments, the nucleic acid is a complementary DNA and further comprises a promoter operably linked to said region, or the complement thereof, encoding said sodium channel  $\beta$ 1A subunit. More particularly, the nucleic acid may comprise a polyadenylation signal operably linked to said region encoding said sodium channel  $\beta$ 1A subunit encoding region. In additional embodiments, the nucleic acid

may also comprise an origin of replication. The nucleic acid may be a viral vector selected from the group consisting of retrovirus, adenovirus, herpesvirus, vaccinia virus and adeno-associated virus. More specifically, the nucleic acid is packaged into a viral particle or the nucleic acid may be packaged into a liposome or a dendrimer formulation.

In other aspects, the present invention contemplates an isolated oligonucleotide of between about 10 and about 50 consecutive bases of a nucleic acid, or complementary thereto, encoding a sodium channel  $\beta 1A$  subunit. In particular embodiments, the oligonucleotide may be 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65 consecutive bases in length or longer. In specific aspects these consecutive bases may be derived from the coding region of SEQ ID NO:1.

Also contemplated herein is a sodium channel  $\beta 1A$  subunit-encoding nucleic acid operably linked to a first promoter. Although not being limited to the promoters indicated herein, preferred promoters may be selected from the group consisting of CMV IE, SV40 IE, RSV LTR,  $\beta$ -actin, tetracycline regulatable, ecdysone regulatable, tyrosinase, retrovirus LTR, PGK HIV-1 promoter, and HIV-2 promoter. In addition, the promoters may be cell specific or tissue specific for the particular cell type being employed to express the nucleic acid. The expression construct may be a lentiviral, adenoviral, adeno-associated viral, vaccinia viral, herpes viral or retroviral expression construct. More particularly, the  $\beta 1A$  encoding nucleic acid sequence is as set forth in SEQ ID NO:1. In other embodiments, the  $\beta 1A$  encoding expression construct encodes a protein of SEQ ID NO:2.

Other aspects of the present invention provide an isolated polypeptide encoding a sodium channel  $\beta 1A$  subunit. More particularly, the sodium channel  $\beta 1A$  subunit has the amino acid sequence as set forth in SEQ ID NO:2. In other embodiments, there is provided an isolated peptide having between about 10 and about 50 consecutive residues of a sodium channel  $\beta 1A$  subunit. In specific embodiments, the peptide is conjugated to a carrier molecule. The carrier molecule may be any molecule commonly used to generate antibodies. For example the carrier molecule may be KLH and BSA. In specific embodiments, the sodium channel  $\beta 1A$  subunit has the amino acid sequence as set forth in SEQ ID NO:2.

Other aspects of the invention provide a monoclonal antibody that binds immunologically to a sodium channel  $\beta 1A$  subunit. In particularly preferred embodiments, the antibody does not bind immunologically to other sodium channel subunit polypeptides. In specific embodiments, the antibody further comprises a detectable label. The label may be selected from the group consisting of a fluorescent label, a chemiluminescent label, a radiolabel and an enzyme. Also provided by the present invention is a hybridoma cell that produces a monoclonal antibody that binds immunologically to a sodium channel  $\beta 1A$  subunit. In preferred embodiments, the antibody does not bind immunologically to other sodium channel subunit polypeptides. In addition the present invention provides a polyclonal antisera, antibodies of which bind immunologically to a sodium channel  $\beta 1A$  subunit. The antisera may be generated from any animal normally used by those of skill in the art to produce antisera. For example the antisera is derived from an animal selected from the group consisting human, mouse, horse, dog, goat, rabbit, rat, and sheep.

Additional embodiments of the present invention provide a method of screening for a modulator of sodium channel activity comprising providing a cell co-expressing a sodium channel  $\beta 1A$  subunit polypeptide with a sodium channel  $\alpha$  subunit; contacting said cell with a candidate modulator substance; and determining the effect of said candidate substance on the sodium channel function in said cell.

In specific embodiments, the determining comprises comparing the sodium current density of the cell in the presence of said candidate substance with sodium current density of said cell in the absence of said candidate substance wherein an alteration of said density is indicative of said candidate substance being a modulator. More particular embodiments describe the candidate substance as being selected from a small molecule library. In preferred embodiments, the candidate substance alters the expression of said sodium  $\beta 1A$  subunit. The method is such that the cell may be contacted *in vitro* or *in vivo*.

Other embodiments describe a modulator of sodium channel activity identified by a method comprising providing a cell co-expressing a sodium channel  $\beta 1A$  subunit polypeptide with a sodium channel  $\alpha$  subunit; contacting said cell with a candidate modulator substance; and determining the effect of said candidate substance on the sodium channel function in said cell. In preferred embodiments, the modulator is an inhibitor of sodium channel  $\beta 1A$  subunit activity. In other embodiments, the modulator

is an activator of sodium channel  $\beta 1A$  subunit activity. In still additional embodiments, the modulator is an inhibitor of sodium channel activity. In specific embodiments, the candidate substance modulates the expression of sodium channel  $\beta 1A$  subunit. In certain other preferred embodiments the modulator is an activator of sodium channel activity. The modulator may be a naturally occurring modulator of sodium channel  $\beta 1A$  activity or is a modulator synthesized from rational drug design.

Other aspects of the present invention contemplate a method for transforming a cell comprising contacting the cell with a nucleic acid expression construct (i) encoding a sodium channel  $\beta 1A$  subunit and (ii) a promoter active in said cell, wherein said promoter is operably linked to the region encoding said sodium channel  $\beta 1A$  subunit, under conditions permitting the uptake of said expression construct by said cell. In particular embodiments, the cell is a brain cell, lung cell, muscle cell, adrenal cell, fibroblast cell, or a cardiac cell. Of course these are merely exemplary and those of skill in the art may use any cell routinely employed in such transformations. In preferred embodiments, the expression construct is encapsulated in a liposome or a dendrimer. In other embodiments, the expression construct is a viral vector selected from the group consisting of retrovirus, adenovirus, adeno-associated virus, vaccinia virus and herpesvirus. In particularly preferred aspects the nucleic acid is encapsulated in a viral particle.

Also provided is a method for decreasing the number of febrile seizures in an individual comprising administering to said individual a modulator of a sodium channel  $\beta 1A$  subunit in an amount effective to change the sodium channel activity in said individual. In preferred embodiments, the modulator is identified according to a method comprising providing a cell expressing a sodium channel  $\beta 1A$  subunit polypeptide; contacting said cell with a candidate substance; and determining the effect of said candidate substance on the activity of said sodium channel  $\beta 1A$  subunit. In specific embodiments, the modulator is an anti-epileptic agent. In other embodiments, the modulator is an inhibitor of sodium channel  $\beta 1A$  subunit. In more particular aspects of the invention the modulator affect the level of expression of the sodium channel  $\beta 1A$  subunit. More specifically, the modulator decreases the expression of sodium channel  $\beta 1A$  subunit

Also provided is a method for treating fibrillar seizures in a subject comprising altering the activity or level of sodium channel  $\beta 1A$  subunits of a cell in said subject. In preferred embodiments, the altering comprises contacting said cell with a sodium channel  $\beta 1A$  subunit under conditions permitting the uptake of said sodium channel  $\beta 1A$  subunit by said cell. In specific embodiments, the contacting comprises contacting said cell with a nucleic acid expression construct (i) encoding a sodium channel  $\beta 1A$  subunit and (ii) a promoter active in said cell, wherein said promoter is operably linked to the region encoding said sodium channel  $\beta 1A$  subunit, under conditions permitting the uptake of said expression construct by said cell. In preferred embodiments, the subject is a human. In other embodiments, it would be useful to decrease the expression of a sodium channel  $\beta 1A$  subunit by for example contacting said cell with a nucleic acid expression construct encoding sodium channel  $\beta 1A$  subunit positioned antisense to a promoter active in said cell, wherein said promoter is operably linked to the region encoding said sodium channel  $\beta 1A$  subunit, under conditions permitting the uptake of said expression construct by said cell.

Another embodiment contemplates a method for decreasing neuropathic pain in an individual comprising administering to said individual a modulator of a sodium channel  $\beta 1A$  subunit in an amount effective to change the sodium channel activity in said individual. In specific embodiments, the modulator decreases the expression of sodium channel  $\beta 1A$  subunit of the cells of said individual. In other embodiments, there is provided a method for treating neuropathic pain in a subject comprising altering the activity or level of sodium channel  $\beta 1A$  subunits of a cell in said subject. Similarly, sodium channel  $\beta 1A$  is involved in certain cardiac functions relating to the sodium channel. As such it is contemplated that methods similar to those set forth for neuropathic pain also could be employed to ameliorate disorders in cardiac function, *e.g.*, cardiac arrhythmia and the like. In preferred embodiments, it is contemplated that altering the activity or level of sodium channel  $\beta 1A$  subunits of a cell in a subject would be useful in treating cardiac arrhythmia.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various



changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

5           **FIG. 1A-FIG. 1D. Sequence analysis of  $\beta$ 1A** FIG. 1A. Deduced amino acid sequence of  $\beta$ 1A. Solid box: signal peptide that becomes cleaved in the mature protein. Arrow: site of alternate splice resulting in retention of intron 3. Astericks: N-linked glycosylation sites. Dotted box: transmembrane segment predicted from Kyte-Doolittle hydropathy profile. Solid underline: amino acid sequence used to synthesize  $\beta$ 1A-MAP. FIG. 1B. Sequence analysis of  $\beta$ 1A - Comparison of  $\beta$ 1A and  $\beta$ 1 amino acid sequences. Upper sequence:  $\beta$ 1A. Lower sequence:  $\beta$ 1. FIG. 1C. Sequence analysis of  $\beta$ 1A - Putative membrane topologies of  $\beta$ 1A and  $\beta$ 1.  $\beta$ 1A is predicted to encode a type I membrane protein with similar topology to  $\beta$ 1. The disulfide-linked immunoglobulin fold (from the indicated carboxy-terminal disulfide bond to the amino terminus; traced by "Ψ") is common to both  $\beta$ 1 and  $\beta$ 1A. Following the alternate splice site (arrow),  $\beta$ 1A contains a novel 66-amino acid juxtamembrane region, 19-amino acid transmembrane segment, and 39-amino acid intracellular domain. FIG. 1D. Sequence analysis of  $\beta$ 1A - Sequence homology to known proteins. Results of BLAST-P search of the Swissprot database using the novel, carboxy-terminal domain of  $\beta$ 1A beginning at residue 130 as the query sequence.

20           **FIG. 2A-FIG. 2D. Effects of  $\beta$ 1A on the functional properties of whole cell sodium currents.** FIG. 2A. Voltage-dependent sodium currents recorded in a SNaIIA cell (top traces) and a SNaIIA $\beta$ 1A cell (bottom traces). Currents were elicited by depolarizations to -40, -30, -20, -10, 0 and +10 mV, from a prepulse potential of -100 mV. FIG. 2B. Mean activation (filled symbols) and inactivation (open symbols) curves for cell lines SNaIIA (circles), SNaIIA $\beta$ 1A-7 (squares), SNaIIA $\beta$ 1A-8 (triangles) and SNaIIA $\beta$ 1A-16 (inverted triangles). For each cell, activation and inactivation were analyzed as described in Methods. The symbols show means of the activation and inactivation data for the different cell lines. In this and subsequent electrophysiology figures, error bars indicate standard errors of the means (SEM). The smooth lines were generated with the Boltzman equation (see Methods), using mean

values of  $V_{1/2}$  and  $k$  determined for each cell line, from fits of individual experiments. Mean values for  $V_{1/2}$  and  $k$  and the number of experiments for each cell line are as follows: activation: SNaIIA,  $V_{1/2} = -11.0 \pm 0.96$ ,  $k = -8.3 \pm 0.28$ ,  $n = 15$ ; SNaIIA $\beta$ 1A-7,  $-8.1 \pm 2.13$ ,  $-7.9 \pm 0.48$ , 6; SNaIIA $\beta$ 1A-8,  $-11.6 \pm 1.21$ ,  $-7.4 \pm 0.31$ , 11; SNaIIA $\beta$ 1A-16,  $-17.0 \pm 1.41$ ,  $-7.18 \pm 0.39$ , 11; inactivation: SNaIIA,  $V_{1/2} = -48.3 \pm 0.77$ ,  $k = 6.5 \pm 0.20$ ,  $n = 13$ ; SNaIIA $\beta$ 1A-7,  $-45.2 \pm 1.25$ ,  $6.8 \pm 0.22$ , 6, SNaIIA $\beta$ 1A-8,  $-45.9 \pm 0.63$ ,  $6.5 \pm 0.21$ ,  $n = 11$ ; SNaIIA $\beta$ 1A-16,  $-47.2 \pm 0.50$ ,  $6.5 \pm 0.15$ , 10. FIG. 2C. Inactivation time constants ( $\tau_{\text{inactivation}}$ ) determined from fits of current decay for SNaIIA (O), SNaIIA $\beta$ 1A-7 ( $\square$ ), SNaIIA $\beta$ 1A-8 ( $\Delta$ ) and SNaIIA $\beta$ 1A-16 ( $\nabla$ ), plotted as a function of test potential. FIG. 2D. Mean  $V_{1/2}$  values for activation (filled symbols) and inactivation (open symbols) for cell lines SNaIIA, SNaIIA $\beta$ 1A-7, SNaIIA $\beta$ 1A-8, SNaIIA $\beta$ 1A-16.

**FIG. 3A and FIG. 3B. Effect of  $\beta$ 1A on the level of expression of functional sodium channels.** FIG. 3A. Current densities for SNaIIA and SNaIIA $\beta$ 1A cell lines. Currents were elicited by depolarization to +10 mV from a prepulse potential of -100 mV. Peak current amplitude was divided by cell capacitance to give current density. Cell capacitance was determined by integrating the area under transients elicited by 3 mV voltage steps applied before series resistance compensation and capacitive transient cancellation. FIG. 3B. Amplitude-frequency histogram for SNaIIA (black bars) and SNaIIA $\beta$ 1A (white bars; data for all three SNaIIA $\beta$ 1A cell lines were combined). Currents were evoked by depolarization to +10 mV. The bars indicate the number of cells with peak currents that fell within different amplitude ranges.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention describes the isolation, molecular cloning and functional characterization of a new protein involved in voltage-gated sodium channel formation. The data presented herein show that  $\beta$ 1A is a splice variant of the  $\beta$ 1 gene.  $\beta$ 1A contains an identical amino terminal and extracellular Ig-fold region as the  $\beta$ 1 protein; however, this region is followed by a significantly different extracellular

juxtamembrane domain, predicted transmembrane region, and predicted intracellular COOH-terminal domain.

The inventors show that the  $\beta 1A$  mRNA is expressed early in embryonic brain development and then disappears after birth. Western blot analysis of membrane preparations using an antibody to a unique, extracellular region of  $\beta 1A$  not found in  $\beta 1$  showed that  $\beta 1A$  protein is expressed in adult rat heart, skeletal muscle, and adrenal gland but was not detected in adult brain or spinal cord.

Immunocytochemical analysis of  $\beta 1A$  expression in adult rat tissues revealed high expression in heart and dorsal root ganglion (DRG) and selective expression in some areas of the brain and spinal cord.  $\beta 1A$  functions to increase channel expression at the plasma membrane when coexpressed with  $\alpha IIA$  subunits in CHL fibroblasts. Unlike  $\beta 1$ , however, mean steady state inactivation curves for  $\alpha \beta 1A$ -expressing cell lines were shifted to more positive potentials than the mean inactivation curves for cells expressing  $\alpha$  alone. Previous studies showed that coexpression of  $\alpha$  and  $\beta 1$  subunits in CHL cells shifted the voltage-dependence of inactivation to more negative potentials compared to  $\alpha$  alone (Isom *et al.*, 1995b). Therefore, the novel, carboxy-terminal domains of  $\beta 1A$  likely is important for electrophysiological function. It has been shown previously that the extracellular domain of  $\beta 1$  is essential for expression and function of the  $\alpha \beta 1$  complex in *Xenopus* oocytes (Chen and Cannon, 1995; McCormick *et al.*, 1998). The inventors suggest that the extracellular Ig fold, common to  $\beta 1$  and  $\beta 1A$ , is essential for the observed increases in channel expression levels. Thus, this report introduces a novel splice variant of  $\beta 1$ ,  $\beta 1A$ , and adds to the understanding of  $\beta 1$  structure/function relationships in terms of channel expression/stabilization and electrophysiology.

The present invention exploits these findings to provide a variety of novel methods and compositions related to voltage-gated sodium channels. The novel protein and DNA sequences described herein and variants thereof may be used in the production of antibodies, expression vectors, in methods for producing recombinant proteins, methods of producing recombinant cells, screening assays and identification of modulators of sodium channels using such assays. In addition, the sodium channel plays a central role in a number of biologically significant areas. For example, the present inventors contemplate that the sodium channel is involved in neuropathic pain

and as such modulators of the sodium channel  $\beta 1A$  subunit may be identified that modulate neuropathic pain. Similarly these channels are important in certain aspects of cardiac function, and the present invention may be employed in various ways of altering, adjusting or otherwise treating disorders stemming from changes in cardiac function. Another interesting observation is that the sodium  $\beta 1A$  subunit may be involved in altering the amount, degree, or severity of fibrillar seizures in an individual. The present invention describes methods and compositions that may be used in modifying, altering or otherwise managing these disorders as well as providing details of the use of the sodium channel  $\beta 1A$  subunit related compositions in various research settings.

### 1. Voltage-Gated Sodium Channels

In order to provide a more detailed understanding of the present invention, the subject of voltage gated sodium channels needs some introduction. As such, the present section provides a background discussion of sodium channels and their function.

The localized cell surface density and the functional properties of sodium channels are a key determinant of the threshold for action potential generation and the frequency of firing of neurons. Myelination of both central and peripheral axons, permits rapid saltatory conduction of action potentials through a cooperation between the axon itself and specialized glial cells which envelope the axon with multiple insulating layers. These cells are the oligodendrocytes in the central nervous system (CNS), and Schwann cells in the peripheral nervous system (PNS). Interruptions in the myelin sheath, known as nodes of Ranvier, contain locally high concentrations of voltage-gated sodium channels, the membrane proteins which are responsible for initiation and propagation of the action potential.

A complex interplay between a number of extracellular and intracellular signaling events is required for proper generation and propagation of an action potential (Salzer 1997; Vabnick and Shrager 1998). It appears that there is an unidentified factor secreted by oligodendrocytes that is necessary for the induction of clustering of CNS sodium channels *in vitro* and *in vivo* (Kaplan *et al.*, 1997). Similarly, Schwann cell contact has been shown to induce sodium channel clustering in PNS axons (Dugandzija-

Novakovic *et al.*, 1995; Joe and Angelides, 1992; Joe and Angelides, 1993). Sodium channels colocalize with the cytoskeletal proteins ankyrin G spectrin at the nodes of Ranvier and there is some evidence to suggest that sodium channels bind ankyrin directly (Srinivasan *et al.*, 1988).

5 Brain and muscle sodium channel  $\alpha$  subunit isoforms interact with multiple members of the syntrophin family, cytoplasmic peripheral membrane proteins which contain a PDZ domain (Gee *et al.*, 1998). Cell adhesion molecules present in the node of Ranvier, such as neurofascin and NrCAM, have also been proposed to participate in linking axonal proteins with glial processes (Davis *et al.*, 1996). Finally, sodium  
10 channel  $\alpha$  subunit isoforms are differentially localized in neurons. For example,  $\alpha$ IIA is located axonally and  $\alpha$ I is located somatodendritically in hippocampal slices (Westenbroek *et al.*, 1989). Thus, information regulating sodium channel localization may be encoded in the amino acid sequence of various subunit isoforms.

Sodium channels isolated from brain are heterotrimeric structures composed of  
15 a central, pore-containing  $\alpha$ -subunit and two auxiliary subunits,  $\beta$ 1 and  $\beta$ 2, which do not form the pore but play critical roles in channel gating, voltage-dependence of activation and inactivation, expression levels, and localization. At least 4 genes encoding sodium channel  $\alpha$  subunits are expressed in the CNS:  $\alpha$ I (Noda *et al.*, 1986a; Noda *et al.*, 1986b),  $\alpha$ II/IIA (Auld *et al.*, 1988; Noda *et al.*, 1986a; Noda *et al.*, 1986b),  
20  $\alpha$ III (Kayano *et al.*, 1988), and  $\alpha$ 6 or Scn8a (Burgess *et al.*, 1995; Schaller *et al.*, 1995). RNA encoding  $\alpha$  subunits is sufficient to direct the synthesis of functional sodium channels in *Xenopus* oocytes (Goldin *et al.*, 1986; Noda *et al.*, 1986b), but co-injection of low molecular weight brain mRNA from brain or skeletal muscle is required for rapid inactivation (Auld *et al.*, 1988; Joho *et al.*, 1990, Krafte *et al.*, 1988; Ukomadu *et al.*, 1992;  
25 Zhou *et al.*, 1991). These results suggested a possible role for the low molecular weight  $\beta$  subunits in sodium channel function. Cloning and functional analysis of the  $\beta$ 1 and  $\beta$ 2 subunits of sodium channels have shown that the  $\beta$ 1 subunit (Bennett *et al.*, 1993; Cannon *et al.*, 1993; Isom *et al.*, 1992, Makita *et al.*, 1994a, Makita *et al.*, 1994b; Schreibmayer *et al.*, 1994, Tong *et al.*, 1993; Wallner *et al.*, 1993) and the  $\beta$ 2 subunit  
30 (Isom *et al.*, 1995b) do indeed strongly modulate sodium channel function.

Glial-derived extracellular matrix molecules, such as tenascin-C (TN-C) and tenascin-R (TN-R), play important roles in cell interactions in the developing nervous

system, such as neuronal migration, neuritogenesis, and neuronal regeneration (Bartsch, 1996; Chiquet-Ehrismann *et al.*, 1994; Erickson, 1993; Schachner *et al.*, 1994). TN-R is expressed predominantly by oligodendrocytes during the onset and early phases of myelin formation, and remains expressed by some oligodendrocytes in the adult (Bartsch *et al.*, 1993; Fuss *et al.*, 1991; Fuss *et al.*, 1993; Pesheva *et al.*, 1989; Wintergerst *et al.*, 1993) as well as some neurons and interneurons in the spinal cord, retina, cerebellum, and hippocampus (Bartsch *et al.*, 1993; Fuss *et al.*, 1993; Wintergerst *et al.*, 1993). Interestingly, TN-R co-localizes with other glial-derived molecules, such as myelin-associated glycoprotein (MAG) and a phosphacan-related molecule, at high density in myelinated CNS nerves (Xiao *et al.*, 1997). TN-R is a multifunctional molecule that promotes neurite outgrowth when presented as a uniform substrate, inhibits growth cone advance when offered as a sharp substrate boundary, and induces axonal defasciculation *in vitro*, resulting from interaction with one of its neuronal receptors, F3/contactin (Lochter and Schachner, 1993; Lochter *et al.*, 1994; Pesheva *et al.*, 1993, Taylor *et al.*, 1993).

Cell adhesion molecules of the Ig superfamily interact homophilically and heterophilically to transduce signals between adjacent cells or adjacent axons where they participate in axonal fasciculation. Cell adhesion molecules of the L1 family have been studied extensively. For example, transfection of neuroglian, a member of the L1 family, into *Drosophila* S2 cells results in homophilic binding and cellular aggregation (Hortsch *et al.*, 1998; Malhotra *et al.*, 1998). It appears that the  $\beta$  subunits behave as classical CAMs in addition to playing roles as channel modulators.  $\beta$  subunits participate in modulation of the voltage-dependence of channel activation and inactivation, channel gating mode, as well as channel expression levels at the plasma membrane.

The  $\beta$  subunits are members of the Ig superfamily and play roles in cellular adhesion and repulsion. Thus, sodium channel  $\beta$  subunits are multifunctional proteins with possible roles independent of the ion channel complex. The inventors propose that the sodium channel  $\beta$  subunits function as true CAMs, acting as bridges between the extra- and intracellular neuronal environments. The present invention provides detailed characterization, cloning and sequencing of an important and novel sodium channel  $\beta$  subunit.

## 2. $\beta$ 1A Protein

According to the present invention, there has been identified a splice variant of the sodium channel  $\beta$ 1 subunit, that results from an intron retention in the  $\beta$ 1 subunit encoding gene. This splice variant is referred to herein as  $\beta$ 1A. Co-expression of  $\beta$ 1A with an  $\alpha$ -subunit results in an increase in sodium current density compared to cells expressing  $\alpha$  alone. This increase in current density reflected two effects of  $\beta$ 1A: 1) an increase in the proportion of cells expressing detectable sodium currents, and 2) an increase in the level of functional sodium channels in expressing cells.

In addition to the entire  $\beta$ 1A molecule, the present invention also relates to fragments of the polypeptide that may or may not retain the  $\alpha$ -subunit regulatory (or other) activity of  $\beta$ 1A. Fragments, including the N-terminus of the molecule may be generated by genetic engineering of translation stop sites within the coding region (discussed below). Alternatively, treatment of the  $\beta$ 1A molecule with proteolytic enzymes, known as proteases, can produce a variety of N-terminal, C-terminal and internal fragments. Examples of fragments may include contiguous residues of the  $\beta$ 1A sequence given in FIG. 1B of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 100, 200, or more amino acids in length. These fragments may be purified according to known methods, such as precipitation (*e.g.*, ammonium sulfate), HPLC, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size separations (sedimentation, gel electrophoresis, gel filtration).

### A. Structural Features

The gene for  $\beta$ 1A encodes a 272 amino acid polypeptide. The predicted molecular weight of this molecule is 50kDa, with a resulting pI of 6.42. Thus, at a minimum, this molecule may be used as a standard in assays where molecule weight and pI are being examined.

$\beta$ 1A, a splice variant of  $\beta$ 1, is the result of the retention of intron 3 containing an in frame stop codon. This alternate splicing event produces a novel carboxy-terminus that includes an extracellular region, a transmembrane segment, and a short intracellular domain. Western blot analysis showed  $\beta$ 1A immunoreactive peptides of

approximately 50 kDa expressed in heart, skeletal muscle, and adrenal gland, but not in adult brain or spinal cord.

A number of cases of intron retention have been reported in the literature, including alternative splicing of the genes encoding leukocyte-common antigen-related protein tyrosine phosphatase (LAR), CD44, effector cell protease receptor-1 (EPR-1), the microtubule-associated protein tau, thyrotropin-releasing hormone receptor, and bovine growth hormone (Tabiti *et al.*, 1996; Higashikawa *et al.*, 1996; Zhang and Longo, 1995; Altieri, 1994; Sadot *et al.*, 1994; de la Pene, *et al.*, 1992; Hampson *et al.*, 1989). In many cases, the retained intron contains an alternate, in-frame termination codon as well as a polyadenylation signal.

Alternative splicing which results in retention of the intron in the primary transcript thus results in an isoform of the protein containing a novel carboxy terminus. Interestingly, this is not the first report of intron retention in the  $\beta 1$  gene. Waxman and coworkers have previously reported that intron 5 of  $\beta 1$  can be retained, creating a novel isoform which is expressed in rat brain, optic nerve, sciatic nerve, and skeletal muscle. This isoform contains a 86 nucleotide insert encoded by intron 5 in the 3' untranslated region (Oh and Waxman, 1994; Dib-Hajj and Waxman, 1995). While the intron retention reported previously does not alter the  $\beta 1$  coding sequence, the present data describe a very significant coding sequence change resulting in a novel carboxy terminus.

Sodium channel  $\beta$  subunits are members of the immunoglobulin (Ig) superfamily and contain extracellular cell adhesion molecule (CAM) domains. Because CAMs interact with extracellular protein ligands as well as with other CAMs, the  $\beta$  subunits likely act in a similar fashion. The inventors suggest that the transmembrane and/or the intracellular domains of the sodium channel  $\beta$  subunits are responsible for transducing a signal from the extracellular environment to cytoskeletal or signaling molecules inside the cell. Tenascin-R (TN-R), an extracellular matrix protein that is secreted by oligodendrocytes during formation of CNS nodes of Ranvier, is a functional modulator of sodium channel  $\beta$  and  $\alpha$  subunits of TN-R binding and can be used to study the effects of varying the subunit sequence.

Given the disclosure of the present invention, it is now possible to construct and express truncation mutations that eliminate the intracellular COOH-terminal domains of the  $\beta$  subunit, introduction of a COOH-terminal signal peptide which eliminates the



transmembrane domain and adds a glycosphosphatidylinositol (GPI) lipid anchor to the  $\beta$  subunit, and point mutations of amino acids in  $\beta$  subunits that are predicted to be located in the extracellular Ig fold. These mutations then are tested in the cell migration assay for modulation by TN-R.

5           The Ig loop region of the  $\beta 1A$  and the other  $\beta$  subunits is responsible for mediating homophilic binding and the intracellular COOH-terminal regions of these molecules are then responsible for transducing a signal resulting in ankyrin recruitment to the plasma membrane. Thus, a truncated  $\beta$  subunit lacking the COOH-terminal intracellular domain is predicted to display homophilic binding but be incapable of  
10           ankyrin recruitment. Given the disclosure presented herein it is now possible to construct mutants to test this *Drosophila* S2 cells. Further, recombinant fragments of TN-R can be used to inhibit homophilic binding between  $\beta$  subunits expressed in S2 cells. This will allow determination of whether these two binding events are mediated by the same or different extracellular amino acid motifs in the  $\beta$  subunits.

15           Some CAMs belonging to the Ig superfamily interact heterophilically with other CAMs to transduce signals across the plasma membrane to the cytoskeleton and other signaling molecules. This interaction can be intercellular or *trans*, such that different CAMs on adjacent cells interact, or it can be intracellular or *cis*, such that adjacent molecules on the same plasma membrane interact to produce a signal. Neurofascin,  
20           ankyrin G, and sodium channels have been shown to be colocalized at CNS nodes of Ranvier. Thus, neurofascin and  $\beta$  subunits may interact via *cis* heterophilic binding.

          Using the *Drosophila* S2 cell model system it will be possible to test for aggregation and ankyrin recruitment by mixing  $\beta 1$ -,  $\beta 2$ -, and/or neurofascin-transfected cells, after one of the cell lines has been labeled with DiI. By performing  
25           coimmunoprecipitations on cells that are cotransfected with a  $\beta$  subunit plus neurofascin it will be possible to test for *cis* interactions. A mutation in the Ig loop region of  $\beta 1$  has recently been implicated in febrile seizures and generalized epilepsy (Wallace *et al.*, 1998). Mutations in L1-CAM are linked to hydrocephalus, motor neuron defects, agencsis of the corpus callosum, and the cerebrospinal tract. Mutations in  
30           myelin P o, a CAM with homology to  $\beta 1$ , may be involved in Charcot-Marie-Tooth disease. Alterations in sodium channel clustering and localization are inherent in demyelinating disease such as multiple sclerosis. Given the present disclosure of a

novel  $\beta$  subunit of the sodium channel containing a COOH terminus that is different from those described for  $\beta 1$  and  $\beta 2$ , there will be an increase the basic understanding of the molecular and cellular biology of sodium channel  $\beta$  subunits, especially their roles as CAMs, and lead to the future development of therapeutic agents for these and related diseases.

### B. Functional Aspects

The most striking functional consequence of  $\alpha IIA$  and  $\beta 1A$  coexpression was a significant increase in sodium current density compared to cells expressing  $\alpha IIA$  subunits alone. This increase in current density reflected two distinct effects of  $\beta 1A$ : 1) an increase in the proportion of cells expressing detectable sodium currents, and 2) an increase in the level of functional sodium channels in expressing cells. Increases in sodium channel expression with  $\beta 1A$  are similar to previous results obtained with the adult  $\beta 1$  isoform in both mammalian cells (Isom *et al.*, 1995) and *Xenopus* oocytes (Isom *et al.*, 1992). These observations are consistent with the hypothesis that  $\beta 1$  and  $\beta 1A$  subunits facilitate the expression of sodium channels, and/or stabilize the channels in the plasma membrane and that the molecular basis for this function resides in the extracellular cell adhesion molecule domain common to the two isoforms. The results of the [ $^3H$ ]-STX binding experiments further support this hypothesis. It is proposed that interaction of the extracellular cell adhesion molecule domain (Ig fold) common to  $\beta 1$  and  $\beta 1A$  with  $\alpha$  may be responsible for the observed effects on channel expression levels. Consistent with this interpretation, it has been shown that the extracellular domain of  $\beta 1$  is essential for modulation of both brain and skeletal muscle  $\alpha$  subunits, whereas the intracellular carboxy-terminal domain is not. Truncated  $\beta 1$  subunits lacking the intracellular carboxy-terminus are fully functional in terms of kinetic modulation of brain and skeletal muscle  $\alpha$  subunits expressed in *Xenopus* oocytes (Chen and Cannon *et al.*, 1995; McCormick *et al.*, 1998). The  $\beta 1$  extracellular domain, together with the residues proximal to the transmembrane domain, constructed in a  $\beta 1/\beta 2$  subunit chimera were found to be sufficient to modify skeletal muscle sodium channel  $\alpha$  subunits expressed in oocytes (Chen and Cannon *et al.*, 1995; McCormick *et al.*, 1998). Finally, residues predicted to be in the Ig fold of  $\beta 1$  interact with type IIA  $\alpha$

subunits (McCormick *et al.*, 1998). Thus, the extracellular cell adhesion domain common to  $\beta 1$  and  $\beta 1A$  appears to be required for function.

Sodium currents in  $\beta 1A$ -expressing cell lines also exhibited subtle functional differences compared to the parent SNaIIA cell line. For example, inactivation curves in SNaIIA $\beta 1A$  cell lines were shifted to slightly more positive potentials than inactivation curves for SNaIIA cells. This finding differs from previous results showing that coexpression of  $\beta 1$  and  $\alpha IIA$  in CHL cells shifts inactivation to potentials approximately 10 mV more negative than for cells expressing  $\alpha$  alone. However, there is a significant difference in the  $\beta 1$  and  $\beta 1A$  proteins in that the latter contains a novel 55 residue long juxtamembrane portion which may explain this difference in steady state inactivation.

In addition to opposite effects on steady state inactivation, the voltage-dependence of activation and the rate of channel inactivation were also different in one of the three  $\beta 1A$ -expressing cell lines, compared to the parent SNaIIA cell line. Thus, whole cell electrophysiological data suggest that  $\beta 1A$  subunits may subtly modulate various aspects of sodium channel function.

TTX-sensitive sodium channel  $\alpha$  subunits expressed in brain (type I/Scn1a, Smith and Goldin, 1998; type II/Scn2a, Isom *et al.*, 1992; Isom *et al.*, 1995; type III/Scn3a, Patton *et al.*, 1994; type VI/PN4/Scn8a, Smith *et al.*, 1998) and skeletal muscle (SkM 1/Scn4a Wallner *et al.*, 1993) have been shown to be modulated by co-expression of  $\beta 1$  subunits in heterologous systems. In contrast, TTX-resistant sodium channel  $\alpha$  subunits expressed in cardiac myocytes (SkM 2/H1/Scn5a; Qu *et al.*, 1995) and peripheral nerve (PN3/SNS/Scn10a; Sangameswaran *et al.*, 1996; Klugbauer *et al.*, 1995; Akopian *et al.*, 1996) are much less sensitive or insensitive to modulation by  $\beta 1$  when co-expressed either in *Xenopus* oocytes or mammalian cells. It is proposed that tissues in which TTX-resistant channels are expressed may also express novel auxiliary subunits, possibly  $\beta 1A$ . Interestingly, heart, skeletal muscle, and DRG express TTX-sensitive and -resistant sodium channel  $\alpha$  subunits (Dib-Hajj *et al.*, 1998). It is shown in the present study that these tissues also express both  $\beta 1$  and  $\beta 1A$  subunits. Given the findings of the present invention, it will now be possible to determine whether TTX-resistant channels are modulated by  $\beta 1A$ .

When the present application refers to the function of  $\beta 1A$  or "wild-type" activity, it is meant that the molecule in question has the ability to modulate the sodium density of a cell co-expressing the sodium  $\alpha$ -subunit. In addition the cellular signaling mediated by these pores is influenced by the  $\beta 1A$ . Other phenotypes that may be considered to be regulated by the normal  $\beta 1A$  gene product include peripheral nerve sodium channels (*i.e.*, SCN10A, SCN11A); cardiac sodium channels (SCN5A); (thus, neuropathic pain and cardiac arrhythmias such as Long QT may be involved); cell adhesion/repulsion - thus including axonal fasciculation/defasciculation, growth cone guidance, and synaptic remodeling following epileptic seizures. Determination of which molecules possess this activity may be achieved using assays familiar to those of skill in the art. For example, transfer of genes encoding  $\beta 1A$ , or variants thereof, into cells that do not have a functional sodium channel, and hence exhibit impaired anion transport, will identify, by virtue of increasing the sodium current density in these cells, those molecules having  $\beta 1A$  function. In addition the assays using TN-R, ankyrin and/or neurofascin binding discussed herein also may be employed to show  $\beta 1A$  function seeing as these molecules are known to interact with  $\beta$  subunits. Additional studies may be performed comparing such assays from cells co-expressing  $\beta 1A$  subunits and an  $\alpha$ -subunit with cells co-expressing other  $\beta$  subunits (*e.g.*,  $\beta 1$ ,  $\beta 2$  and the like) with an  $\alpha$ -subunit thereby giving a quantitative and qualitative difference in the function/activity of a  $\beta 1A$  as compared to *e.g.*, a  $\beta 1$  or  $\beta 2$  subunit.

As stated above,  $\beta 1A$  is belongs to the Ig superfamily of CAMs.  $\beta 1A$  is predicted to encode a type I membrane protein with similar topology to  $\beta 1$  (FIG. 1C).  $\beta 1A$  contains a novel 66-amino acid juxtamembrane region, 19-amino acid transmembrane segment, and 39-amino acid intracellular domain. The disulfide-linked immunoglobulin fold is common to both  $\beta 1$  and  $\beta 1A$ . The  $\beta$  subunits are known to regulate the function of the pore-forming  $\alpha$ -subunit of the sodium channel. Given that there is a substantial difference in the COOH terminus of the  $\beta 1A$  as compared to *e.g.*  $\beta 1$ , it will be desirable to determine the effect of this novel COOH terminus on the function in the sodium channel-regulating role of  $\beta 1A$ . This also may be a fruitful approach to developing screening assays for the absence of  $\beta 1A$  function or in the development of therapies, for example, in targeting the sodium current density and

other functions of  $\beta 1A$ , targeting the substrate upon which  $\beta 1A$  acts (*e.g.*,  $\alpha$  subunit, ankyrin, TN-R), and the like.

### C. Variants of $\beta 1A$

5 Amino acid sequence variants of the polypeptide can be substitution, insertion or deletion variants. Deletion variants lack one or more residues of the native protein which are not essential for function or immunogenic activity, and are exemplified by the variants lacking a transmembrane, COOH-terminus, the Ig fold or other region described above. Another common type of deletion variant is one lacking secretory signal sequences or  
10 signal sequences directing a protein to bind to a particular part of a cell. Insertion mutants typically involve the addition of material at a non-terminal point in the polypeptide. This may include the insertion of an immunoreactive epitope or simply a single residue. Terminal additions, called fusion proteins, are discussed below.

Substitutional variants typically contain the exchange of one amino acid for  
15 another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, such as stability against proteolytic cleavage, without the loss of other functions or properties. Substitutions of this kind preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes  
20 of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to  
25 tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example,  
30 antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence, and nevertheless obtain a protein with like

properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity, as discussed below. Table 1 shows the codons that encode particular amino acids.

In making such changes, the hydropathic index of amino acids may be considered.

5 The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA,  
10 antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are:  
isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine  
(+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8);  
15 tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5);  
glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids  
20 whose hydrophilicity values are within  $\pm 2$  is preferred, those that are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of  
25 its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0  $\pm$  1);  
glutamate (+3.0  $\pm$  1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0);  
threonine (-0.4); proline (-0.5  $\pm$  1); alanine (-0.5); histidine \*(-0.5); cysteine (-1.0);  
30 methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3);  
phenylalanine (-2.5); tryptophan (-3.4).

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity,

hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

5           Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson *et al.*, "Peptide Turn Mimetics" in *BIOTECHNOLOGY AND PHARMACY*, Pezzuto *et al.*, Eds., Chapman and Hall, New York (1993). The underlying rationale behind the use of peptide mimetics  
10           is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule. These principles may be used, in conjunction with the principles outline above, to engineer second generation molecules having many of the natural properties of  $\beta 1A$ ,  
15           but with altered and even improved characteristics. The generation of specific mutants is described in the examples.

#### **D.     Domain Switching**

As described in the examples, the present inventors have identified that the  $\beta 1A$   
20           is a splice variant of  $\beta 1$  subunit of voltage-gated sodium channels. This provides a starting point for further mutational analysis of the molecule. One way in which this information can be exploited is in "domain switching."

Domain switching involves the generation of chimeric molecules using different but, in many cases, related polypeptides. By comparing the  $\beta 1$  and  $\beta 1A$ , the inventors  
25           have predicted the functionally significant regions of these molecules. It is possible, then, to switch related domains of these molecules in an effort to determine the criticality of these regions to the  $\beta$  subunit activity and function. These molecules may have additional value in that these "chimeras" can be distinguished from natural molecules, while possibly providing the same function. Also it should be possible to  
30           switch the domains of  $\beta 1A$  subunits from voltage dependant sodium channels with those of  $\beta$  subunits from non-voltage dependant sodium channels.

In addition to switching domains between  $\beta 1$  and  $\beta 1A$  it should also be possible to identify the  $\beta 1A$  proteins, if any, from other animals such as rat, rabbit, monkey, gibbon, chimp, ape, baboon, cow, pig, horse, sheep and cat. Upon identification and isolation of these homologs, variants and mutants, and in conjunction with other analyses, certain active or functional domains can be identified. These domains can then be switched with those already identified herein to yield additional information about the conservation of these domains across species.

Based on sequence identity, at the amino acid level, of the  $\beta 1A$  and  $\beta 1$  proteins, it may be inferred that even small changes in the primary sequence of the molecule will affect function. Further analysis of mutations and their predicted effect on secondary structure will add to this understanding.

Another structural aspect of  $\beta 1A$  that provides fertile ground for domain switching experiments is the Ig fold domain. This domain may be substituted for Ig folds from other members of the Ig superfamily domains in order to alter the specificity of this function. A further investigation of the homology between  $\beta 1A$  and other members of the Ig superfamily is warranted by this observation.

#### **E. Fusion Proteins**

A specialized kind of insertional variant is the fusion protein. This molecule generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion of a second polypeptide. For example, fusions typically employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful fusions include linking of functional domains, such as active sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions. One particular fusion of interest would include a deletion construct lacking the carboxy terminal site of  $\beta 1A$  but containing other regions that could bind the substrate molecule. Indeed any of the distinct regions of the  $\beta 1A$  protein can be removed or replaced in order to determine their function in relation to the whole protein. Fusion to a polypeptide that can be used for purification of the substrate- $\beta 1A$  complex would serve to isolate the substrate for identification and analysis.



## F. Protein Purification

It will be desirable to purify  $\beta$ 1A or variants thereof. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography, polyacrylamide gel electrophoresis, and isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number."

The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

5 Various techniques suitable for use in protein purification are well known to those of skill in the art. These include, for example, precipitation with ammonium sulfate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. It is believed that the  
10 order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified  
15 products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low  
20 pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will  
25 therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can  
30 be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the

concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

5           Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is 10           unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, etc. There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

15           Affinity chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. 20           Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, etc.).

          A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins 25           are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins. Other lectins that have been include lentil lectin, wheat germ agglutinin, which has been useful in the purification of N-acetyl glucosaminyl residues, and *Helix pomatia* lectin. Lectins themselves are purified 30           using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-

galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

### G. Synthetic Peptides

The present invention also describes smaller  $\beta$ 1A-related peptides for use in various embodiments of the present invention. Because of their relatively small size, the peptides of the invention can also be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, (1984); Tam *et al.*, (1983); Merrifield, (1986); and Barany and Merrifield (1979). Short peptide sequences, or libraries of overlapping peptides, usually from about 6 up to about 35 to 50 amino acids, which correspond to the selected regions described herein, can be readily synthesized and then screened in screening assays designed to identify reactive peptides. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression.

### H. Antigen Compositions

The present invention also provides for the use of  $\beta$ 1A proteins or peptides as antigens for the immunization of animals relating to the production of antibodies. It is envisioned that either  $\beta$ 1A, or portions thereof, will be coupled, bonded, bound, conjugated or chemically-linked to one or more agents via linkers, polylinkers or derivatized amino acids. This may be performed such that a bispecific or multivalent composition or vaccine is produced. It is further envisioned that the methods used in

the preparation of these compositions will be familiar to those of skill in the art and should be suitable for administration to animals, *i.e.*, pharmaceutically acceptable. Preferred agents are the carriers are keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA).

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### 3. Nucleic Acids encoding $\beta$ 1A Protein

The present invention also provides, in another embodiment, nucleic acid sequences encoding  $\beta$ 1A. The present invention is not limited in scope to these genes, however, as one of ordinary skill in the art could, using these nucleic acids, readily identify related homologs in various other species (*e.g.*, rat, dog, rabbit, monkey, gibbon, chimp, ape, baboon, cow, pig, horse, sheep, cat and other species).

10

In addition, it should be clear that the present invention is not limited to the specific nucleic acids disclosed herein. As discussed below, a " $\beta$ 1A encoding nucleic acid" may contain a variety of different bases and yet still produce a corresponding polypeptide that is functionally indistinguishable, and in some cases structurally, from the polypeptide disclosed herein as SEQ ID NO:2.

15

Similarly, any reference to a nucleic acid should be read as encompassing a host cell containing that nucleic acid and, in some cases, capable of expressing the product of that nucleic acid. In addition to therapeutic considerations, cells expressing nucleic acids of the present invention may prove useful in the context of screening for agents that induce, repress, inhibit, augment, interfere with, block, abrogate, stimulate or enhance the function of  $\beta$ 1A or voltage gated sodium channel activity in general.

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#### A. Nucleic Acids Encoding $\beta$ 1A

The nucleic acid given in FIG. 1C and disclosed in SEQ ID NO:1 represent the  $\beta$ 1A encoding nucleic acids of the present invention. Nucleic acids according to the present invention may encode an entire  $\beta$ 1A protein as shown in SEQ ID NO:2, a domain of  $\beta$ 1A that expresses a particular function attributable to  $\beta$ 1A, or any other fragment of the  $\beta$ 1A sequences set forth herein. The nucleic acid may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organism. In preferred embodiments, however, the nucleic acid would comprise complementary DNA (cDNA). Also contemplated is a cDNA plus a natural intron or an intron derived from another gene; such

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engineered molecules are sometime referred to as "mini-genes." This is particularly of note seeing as the  $\beta$ 1A protein results from a splice variant of  $\beta$ 1 gene. The  $\beta$ 1A protein results from a retention of intron 3 of the  $\beta$ 1 gene. At a minimum, it should be understood that these and other nucleic acids of the present invention may be used as molecular weight standards in, for example, gel electrophoresis.

The term "cDNA" is intended to refer to DNA prepared using messenger RNA (mRNA) as template. The advantage of using a cDNA, as opposed to genomic DNA or DNA polymerized from a genomic, non- or partially-processed RNA template, is that the cDNA primarily contains coding sequences of the corresponding protein. There may be times when the full or partial genomic sequence is preferred, such as where the non-coding regions are required for optimal expression or where non-coding regions such as introns are to be targeted in an antisense strategy.

It also is contemplated that a given  $\beta$ 1A from a given species may be represented by natural variants that have slightly different nucleic acid sequences but, nonetheless, encode the same protein (see Table 1 below).

As used in this application, the term "a nucleic acid encoding a  $\beta$ 1A" refers to a nucleic acid molecule that has been isolated free of total cellular nucleic acid. In preferred embodiments, the invention concerns a nucleic acid sequence essentially as set forth in SEQ ID NO:1. The term "as set forth in SEQ ID NO:1" means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:1. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine (Table 1, below), and also refers to codons that encode biologically equivalent amino acids, as discussed in the following pages.

**TABLE 1**

| <b>Amino Acids</b> |     |   | <b>Codons</b> |     |     |     |     |     |
|--------------------|-----|---|---------------|-----|-----|-----|-----|-----|
| Alanine            | Ala | A | GCA           | GCC | GCG | GCU |     |     |
| Cysteine           | Cys | C | UGC           | UGU |     |     |     |     |
| Aspartic acid      | Asp | D | GAC           | GAU |     |     |     |     |
| Glutamic acid      | Glu | E | GAA           | GAG |     |     |     |     |
| Phenylalanine      | Phe | F | UUC           | UUU |     |     |     |     |
| Glycine            | Gly | G | GGA           | GGC | GGG | GGU |     |     |
| Histidine          | His | H | CAC           | CAU |     |     |     |     |
| Isoleucine         | Ile | I | AUA           | AUC | AUU |     |     |     |
| Lysine             | Lys | K | AAA           | AAG |     |     |     |     |
| Leucine            | Leu | L | UUA           | UUG | CUA | CUC | CUG | CUU |
| Methionine         | Met | M | AUG           |     |     |     |     |     |
| Asparagine         | Asn | N | AAC           | AAU |     |     |     |     |
| Proline            | Pro | P | CCA           | CCC | CCG | CCU |     |     |
| Glutamine          | Gln | Q | CAA           | CAG |     |     |     |     |
| Arginine           | Arg | R | AGA           | AGG | CGA | CGC | CGG | CGU |
| Serine             | Ser | S | AGC           | AGU | UCA | UCC | UCG | UCU |
| Threonine          | Thr | T | ACA           | ACC | ACG | ACU |     |     |
| Valine             | Val | V | GUA           | GUC | GUG | GUU |     |     |
| Tryptophan         | Trp | W | UGG           |     |     |     |     |     |
| Tyrosine           | Tyr | Y | UAC           | UAU |     |     |     |     |

5           Allowing for the degeneracy of the genetic code, sequences that have at least about 50%, usually at least about 60%, more usually about 70%, most usually about 80%, preferably at least about 90% and most preferably about 95% of nucleotides that are identical to the nucleotides of SEQ ID NO:1 will be sequences that are "as set forth in SEQ ID NO:1." Sequences that are essentially the same as those set forth in SEQ ID NO:1 may also be functionally defined as sequences that are capable of hybridizing to a  
10           nucleic acid segment containing the complement of SEQ ID NO:1 under standard conditions.

15           The DNA segments of the present invention include those encoding biologically functional equivalent  $\beta$ 1A proteins and peptides, as described above. Such sequences may arise as a consequence of codon redundancy and amino acid functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-

directed mutagenesis techniques or may be introduced randomly and screened later for the desired function, as described below.

### **B. Oligonucleotide Probes and Primers**

5 Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:1. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementary rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially  
10 complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:1 under relatively stringent conditions such as those described herein.

Complementarity between two single-stranded molecules may be "partial", in which only some of the nucleic acids bind, or it may be complete when total  
15 complementarity exists between the single-stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend upon binding between nucleic acids strands.

20 The term "homology", as used herein, refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is one that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid; it is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely  
25 complementary sequence to the target sequence may be examined using a hybridization assay (Southern or northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous sequence or probe to the target sequence under conditions of low stringency. This is not to say that  
30 conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of



complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding, the probe will not hybridize to the second non-complementary target sequence.

Thus, the sequences of the present invention may encode the entire  $\beta$ 1A protein or functional or non-functional fragments thereof. Alternatively, the hybridizing segments may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more base pairs will be used, although others are contemplated. Longer polynucleotides encoding 250, 500, 1000, 1212, 1500, 2000, 2500, 3000 or 3431 bases and longer are contemplated as well. Such oligonucleotides will find use, for example, as probes in Southern and Northern blots and as primers in amplification reactions.

The term "hybridization", as used herein, refers to any process by which a strand of nucleic acid binds with a complementary strand through base pairing. The term "hybridization complex", as used herein, refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (*e.g.*, membranes, filters, chips, pins or glass slides to which cells have been fixed for *in situ* hybridization).

Suitable hybridization conditions will be well known to those of skill in the art. As known in the art, numerous conditions may be employed to comprise either low or high stringency conditions for hybridization. Factors such as the length and nature (DNA, RNA, base composition) of the sequence, nature of the target (DNA, RNA, base composition, presence in solution or immobilization, etc.), and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate and/or polyethylene glycol) are considered and the hybridization solution may be

varied to generate conditions of either low or high stringency different from, but equivalent to, the above listed conditions.

In certain applications, for example, substitution of amino acids by site-directed mutagenesis, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur even though the sequences of probe and target strand are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 μM MgCl<sub>2</sub>, at temperatures ranging from approximately 40°C to about 72°C. Formamide and SDS also may be used to alter the hybridization conditions.

One method of using probes and primers of the present invention is in the search for genes related to β1A or, more particularly, homologs of β1A from other species. The existence of a rat homolog strongly suggests that other homologs of the human β1A will be discovered in species more closely related, and perhaps more remote, than mouse. Normally, the target DNA will be a genomic or cDNA library, although screening may involve analysis of RNA molecules. By varying the stringency of hybridization, and the region of the probe, different degrees of homology may be discovered.

Another way of exploiting probes and primers of the present invention is in site-directed, or site-specific mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of

mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

The technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, taking into account the degree of mismatch when selecting hybridization conditions, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

### C. Antisense Constructs

In some cases, mutant  $\beta$ 1A proteins may not be non-functional. Rather, they may have aberrant functions that cannot be overcome by replacement gene therapy,

even where the "wild-type" molecule is expressed in amounts in excess of the mutant polypeptide. Antisense treatments are one way of addressing this situation. Antisense technology also may be used to "knock-out" function of  $\beta 1A$  in the development of cell lines or transgenic mice for research, diagnostic and screening purposes.

5           Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and  
10           adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

          Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix  
15           formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell,  
20           either in vitro or in vivo, such as within a host animal, including a human subject.

          Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes  
25           an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included  
30           simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme; see below) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

#### **D. Ribozymes**

Another approach for addressing the "dominant negative" mutant protein is through the use of ribozymes. Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cook, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cook *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cook *et al.*, 1981).

For example, U.S. Patent No. 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

#### 4. Preparation of Vectors for Cloning, Gene Transfer and Expression

Within certain embodiments expression vectors are employed to express the  $\beta$ 1A polypeptide product, which can then be purified and, for example, be used to vaccinate animals to generate antisera or monoclonal antibodies with which further studies may be conducted. In other embodiments, the expression vectors are used in gene therapy. Expression requires that appropriate signals be provided in the vectors, and which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells also are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable cell clones expressing the products are also provided, as is an element that links expression of the drug selection markers to expression of the polypeptide.

##### A. Regulatory Elements

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

In preferred embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the *tk* promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under

the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product.

Promoters that may be useful include insulin, elastin, amylase, pdr-1, pdx-1 and glucokinase, MMTV, MT-1, ecdysone and RuBisco, c-fos, TNF-alpha, C-reactive protein (Arcone *et al.*, 1988), haptoglobin (Oliviero *et al.*, 1987), serum amyloid A2, C/EBP alpha, IL-1, IL-6 (Poli and Cortese, 1989), Complement C3 (Wilson *et al.*, 1990), IL-8, alpha-1 acid glycoprotein (Prowse and Baumann, 1988), alpha-1 antitrypsin, lipoprotein lipase (Zechner *et al.*, 1988), angiotensinogen (Ron *et al.*, 1991), fibrinogen, c-jun (inducible by phorbol esters, TNF-alpha, UV radiation, retinoic acid, and hydrogen peroxide), collagenase (induced by phorbol esters and retinoic acid), metallothionein (heavy metal and glucocorticoid inducible), Stromelysin (inducible by phorbol ester, interleukin-1 and EGF), alpha-2 macroglobulin and alpha-1 antichymotrypsin. Other promoters that could be used according to the present invention include Lac-regulatable, chemotherapy inducible (*e.g.* MDR), and heat (hyperthermia) inducible promoters, Radiation-inducible (*e.g.*, EGR (Joki *et al.*, 1995)), Alpha-inhibin, RNA pol III tRNA met and other amino acid promoters, U1 snRNA (Bartlett *et al.*, 1996), MC-1, PGK, -actin and alpha-globin. Many other promoters that may be useful are listed in Walther and Stein (1996). This list is not intended to be exhaustive of all the possible elements involved in the promotion of gene expression but, merely, to be exemplary thereof.

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized



much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

The list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct is extensive and well known to those in the art. Any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

## **B. Selectable Markers**

In certain embodiments of the invention, the cells contain nucleic acid constructs of the present invention, a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful

selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed.

Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

### C. Multigene Constructs and IRES

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well as an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

### 5. Delivery of Expression Vectors

In order to effect expression of sense or antisense nucleic acid constructs, the expression construct must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo*, as in the treatment of certain disease states. One mechanism for delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Thus, in certain embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kb of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

The viral vectors used herein may be adenoviral, such as described in U.S. Patent No. 5,824,544; U.S. Patent No. 5,707,618; U.S. Patent No. 5,693,509; U.S. Patent No. 5,670,488; U.S. Patent No. 5,585,362 retroviral, such as described in U.S. Patent No. 5,888,502; U.S. Patent No. 5,830,725; U.S. Patent No. 5,770,414; U.S. Patent No. 5,686,278; U.S. Patent No. 4,861,719; an adeno-associated viral, such as described in U.S. Patent No. 5,474,935; U.S. Patent No. 5,139,941; U.S. Patent No. 5,622,856; U.S. Patent No. 5,658,776; U.S. Patent No. 5,773,289; U.S. Patent No. 5,789,390; U.S. Patent No. 5,834,441; U.S. Patent No. 5,863,541; U.S. Patent No. 5,851,521; U.S. Patent No. 5,252,479; an adenoviral-adenoassociated viral hybrid, such as described in U.S. Patent No. 5,856,152; and a vaccinia viral or a herpesviral, such as described in U.S. Patent No. 5,879,934; U.S. Patent No. 5,849,571; U.S. Patent No. 5,830,727; U.S. Patent No. 5,661,033; U.S. Patent No. 5,328,688 vector.

Delivery of the expression constructs through non-viral vectors also is contemplated. Such delivery may employ microinjection (U.S. Patent No. 5,612,205), electroporation (U.S. Patent No. 5,507,724; U.S. Patent No. 5,869,326; U.S. Patent No. 5,824,547; U.S. Patent No. 5,789,213; U.S. Patent No. 5,749,847; U.S. Patent No. 5,019,034; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), calcium phosphate coprecipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990), DEAE dextran introduction (Gopal, 1985), receptor mediated introduction

(Wu and Wu, 1987; Wu and Wu, 1988), liposome mediated introduction (U.S. Patent No. 5,631,018; U.S. Patent No. 5,620,689; U.S. Patent No. 5,861,314; U.S. Patent No. 5,855,910; U.S. Patent No. 5,851,818; U.S. Patent No. 5,827,703, U.S. Patent No. 5,785,987; Nicolau and Sene, 1982; Fraley *et al.*, 1979), dendrimer technology (U.S. Patent 5,795,581; U.S. Patent 5,714,166; U.S. Patent 5,661,025), naked DNA injection (Harland and Weintraub, 1985) and particle bombardment (U.S. Patent No. 5,836,905; U.S. Patent No. 5,120,657; Yang *et al.*, 1990).

Once the expression construct has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

## 6. Cell Culture and Propagation

Primary mammalian cell cultures may be prepared in various ways. In order for the cells to be kept viable while *in vitro* and in contact with the expression construct, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

One embodiment of the foregoing involves the use of gene transfer to immortalized cells for the production of proteins. The gene for the protein of interest may be transferred as described above into appropriate host cells followed by culture of cells under the appropriate conditions. The gene for virtually any polypeptide may be employed in this manner. The generation of recombinant expression vectors, and the

elements included therein, are discussed above. Alternatively, the protein to be produced may be an endogenous protein normally synthesized by the cell in question.

5 Examples of useful mammalian host cell lines are Vero and HeLa cells and cell lines of Chinese hamster ovary, W138, BHK, COS-7, 293, HepG2, NIH3T3, RIN and MDCK cells. In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and process the gene product in the manner desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-  
10 translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to insure the correct modification and processing of the foreign protein expressed.

A number of selection systems may be used including, but not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine  
15 phosphoribosyltransferase genes, in *tk-*, *hgpri-* or *aprt-* cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for *dhfr*, that confers resistance to methotrexate; *gpt*, that confers resistance to mycophenolic acid; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

20 Animal cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

25 Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. However, suspension cultured cells have limitations, such as tumorigenic potential and lower protein production than adherent cells.

30 Large scale suspension culture of mammalian cells in stirred tanks is a common method for production of recombinant proteins. Two suspension culture reactor designs are in wide use - the stirred reactor and the airlift reactor. The stirred design has successfully been used on an 8000 liter capacity for the production of interferon. Cells are grown in a stainless steel tank with a height-to-diameter ratio of 1:1 to 3:1. The culture is usually mixed with one or more agitators, based on bladed disks or

marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through seals on stirrer shafts.

5           The airlift reactor, also initially described for microbial fermentation and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design  
10 is the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easily, has good mass transfer of gases and generates relatively low shear forces.

          The antibodies of the present invention are particularly useful for the isolation of antigens by immunoprecipitation. Immunoprecipitation involves the separation of  
15 the target antigen component from a complex mixture, and is used to discriminate or isolate minute amounts of protein. For the isolation of membrane proteins cells must be solubilized into detergent micelles. Nonionic salts are preferred, since other agents such as bile salts, precipitate at acid pH or in the presence of bivalent cations. Antibodies and their uses are discussed further, below.

## 20           7.       **Generating Antibodies Reactive With $\beta$ 1A**

          In another aspect, the present invention contemplates an antibody that is immunoreactive with a  $\beta$ 1A molecule of the present invention, or any portion thereof. An antibody can be a polyclonal or a monoclonal antibody. In a preferred embodiment,  
25 an antibody is a monoclonal antibody. Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Harlow and Lane, 1988).

          Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the  
30 production of antisera. Typically an animal used for production of antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the

relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for isoforms of antigen may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of the compounds of the present invention can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against the compounds of the present invention. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and Western blot methods and in immunohistochemical procedures such as tissue staining, as well as in other procedures which may utilize antibodies specific to  $\beta 1A$ -related antigen epitopes. Additionally, it is proposed that monoclonal antibodies specific to the particular  $\beta 1A$  of different species may be utilized in other useful applications

In general, both polyclonal and monoclonal antibodies against  $\beta 1A$  may be used in a variety of embodiments. For example, they may be employed in antibody-based cloning protocols to obtain cDNAs or genes encoding other  $\beta 1A$ . They may also be used in inhibition studies to analyze the effects of  $\beta 1A$  related peptides in cells or animals. Anti- $\beta 1A$  antibodies will also be useful in immunolocalization studies to analyze the distribution of  $\beta 1A$  during various cellular events, for example, to determine the cellular or tissue-specific distribution of  $\beta 1A$  polypeptides under different points in the cell cycle. A particularly useful application of such antibodies is in purifying native or recombinant  $\beta 1A$ , for example, using an antibody affinity column. The operation of all such immunological techniques will be known to those of skill in the art in light of the present disclosure.

Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Harlow and Lane, 1988). More specific examples of monoclonal antibody preparation are give in the examples below.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as

may be achieved by coupling a peptide or polypeptide immunogen to a carrier.

Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, *m*-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As also is well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified  $\beta$ 1A protein, polypeptide or peptide or cell expressing high levels of  $\beta$ 1A. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep, or frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.



Following immunization, somatic cells with the potential for producing antibodies, specifically B-lymphocytes (B-cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately  $5 \times 10^7$  to  $2 \times 10^8$  lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with cell fusions.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Milstein, 1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, around  $1 \times 10^{-6}$  to  $1 \times 10^{-8}$ . However, this does not pose a problem, as the viable, fused hybrids

are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B-cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the mAbs are naturally secreted into the culture medium from which they can be

readily obtained in high concentrations. mAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

## 8. Methods for Screening Active Compounds

The present invention also contemplates the use of  $\beta 1A$  and active fragments, and nucleic acids coding thereof, in the screening of compounds for sodium channel modulator activity. Such activity may be a stimulatory activity in which the activity of the sodium channel is increased or an inhibitory activity in which the activity of the sodium channel is decreased. In certain instances, the modulator may be active in stimulating  $\beta 1A$  activity, overcoming the lack of  $\beta 1A$  or blocking the effect of a mutant  $\beta 1A$  molecule. These assays may make use of a variety of different formats and may depend on the kind of "activity" for which the screen is being conducted. Contemplated functional "read-outs" include binding to a compound, inhibition of binding to a substrate, ligand, receptor or other binding partner by a compound, or a functional readout such as monitoring sodium current density.

To date there are a number of agents known to alter the activity of sodium channels. These agents include but are not limited to blockers and activators of sodium channels. The present section is directed to identifying additional modulators of sodium channel function using the findings of the present invention.

Useful compounds in this regard will not be limited to those mentioned above. The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. However, prior to testing of such compounds in humans or animal models, it may be necessary to test a variety of candidates to determine which have potential.

Accordingly, in screening assays to identify useful agents which modulate sodium channel function, it is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds.

In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to modulate the activity of the sodium channel, the method including generally the steps of

- (a) obtaining a cell co-expressing a  $\beta 1A$  channel subunit with a sodium channel  $\alpha$ -subunit;
- (b) admixing a candidate substance with the cell; and
- (c) determining the ability of the candidate substance to alter the sodium channel function of the cell.

To identify a candidate substance as being capable of modulating sodium channel function, one would measure or determine the sodium current density of the cell that co-expresses a  $\beta 1A$  channel subunit with a sodium channel  $\alpha$ -subunit in the absence of the added candidate substance. One would then add the candidate substance to the cell and re-determine the sodium current density in the presence of the candidate substance. A candidate substance which produces an alteration in the sodium current density relative to the density in its absence is indicative of a candidate substance with modulatory capability.

The candidate screening assay is quite simple to set up and perform, and is related in many ways to the assay discussed above for determining sodium current density. Thus, after obtaining an suitable test cell, one will admix an effective amount of candidate substance with the cell, under conditions which would allow the sodium channel to function.

"Effective amounts", in certain circumstances, are those amounts effective at reproducibly altering sodium current density in comparison to the normal levels in the absence of the candidate substance. Compounds that achieve significant appropriate changes in activity will be used. If desired, a battery of compounds may be screened *in vitro* to identify other agents for use in the present invention.

A significant alteration in the sodium channel function, *e.g.*, as measured using sodium current density, are represented by an increase/decrease in sodium current density levels of at least about 30%-40%, and most preferably, by changes of at least about 50%, with higher values of course being possible.

Quantitative *in vitro* testing of the inhibitor is not a requirement of the invention as it is generally envisioned that the agents will often be selected on the basis of their known properties or by structural and/or functional comparison to those agents already

demonstrated to be effective. Therefore, the effective amounts will often be those amounts proposed to be safe for administration to animals in another context, for example, as disclosed herein.

5                   **A.     *In vitro* Testing of Identified Compounds**

In certain in vitro assays, the agents identified may be those which bind to the  $\beta$ 1A molecule or fragment thereof. The polypeptide or fragment may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the polypeptide or the compound may be labeled, thereby permitting determining of  
10                   binding.

In another embodiment, the assay may measure the inhibition of binding of  $\beta$ 1A to a natural or artificial substrate or binding partner. Competitive binding assays can be performed in which one of the agents ( $\beta$ 1A, binding partner or compound) is labeled. Usually, the polypeptide will be the labeled species. One may measure the amount of  
15                   free label versus bound label to determine binding or inhibition of binding.

Another technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with  $\beta$ 1A and washed. Bound polypeptide is detected by various methods.

20                   Purified  $\beta$ 1A can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to the polypeptide can be used to immobilize the polypeptide to a solid phase. Also, fusion proteins containing a reactive region (preferably a terminal region) may be used to link the  $\beta$ 1A active region to a solid phase.

25                   Various cell lines containing wild-type or natural or engineered mutations in  $\beta$ 1A can be used to study various functional attributes of  $\beta$ 1A and how a candidate compound affects these attributes. Methods for engineering mutations are described elsewhere in this document. In such assays, the compound would be formulated appropriately, given its biochemical nature, and contacted with a target cell.

30                   Depending on the assay, culture may be required. The cell may then be examined by virtue of a number of different physiologic assays. Alternatively, molecular analysis may be performed in which the function of  $\beta$ 1A, or related pathways, may be explored.

This may involve assays such as those for protein expression, enzyme function, substrate utilization, mRNA expression (including differential display of whole cell or polyA RNA) and others.

5                    **B.      *In Vivo* Assays**

The present invention also encompasses the use of various animal models. Here, the identity seen between human and rat  $\beta 1A$  provides an excellent opportunity to examine the function of  $\beta 1A$  in a whole animal system where it is normally expressed.

10                    Treatment of animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route the could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous,  
15 intramuscular, intraperitoneal or intravenous injection. Specifically contemplated are systemic intravenous injection, regional administration via blood or lymph supply and intratumoral injection.

**C.      Rational Drug Design**

20                    The goal of rational drug design is to produce structural analogs of biologically active polypeptides or compounds with which they interact (agonists, antagonists, inhibitors, binding partners, *etc.*). By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different susceptibility to alteration or which may affect the function of various other molecules.  
25 In one approach, one would generate a three-dimensional structure for  $\beta 1A$  or a fragment thereof. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches. An alternative approach, "alanine scan," involves the random replacement of residues throughout molecule with alanine, and the resulting affect on function determined.

30                    It also is possible to isolate a  $\beta 1A$  specific antibody, selected by a functional assay, and then solve its crystal structure. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a

functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotypic would be expected to be an analog of the original antigen. The anti-idiotypic could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

Thus, one may design drugs which have improved  $\beta 1A$  activity or which act as stimulators, inhibitors, agonists, antagonists or  $\beta 1A$  or molecules affected by  $\beta 1A$  function. By virtue of the availability of cloned  $\beta 1A$  sequences, sufficient amounts of  $\beta 1A$  can be produced to perform crystallographic studies. In addition, knowledge of the polypeptide sequences permits computer employed predictions of structure-function relationships.

#### **D. Known Modulators of Sodium Channel Function**

As stated above, it may be that known modulators of sodium channel function are employed as a starting point to generate new and novel compounds that would be useful as blockers or activators of sodium channel function.

Sodium channel blockers are a well characterized group of pharmaceutical agents especially in the control of arrhythmia. These drugs include but are not limited to quinidine, phenytoin, mexiletine, tocainide, procainamide, disopyramide, moricizine, propafenone, flecainide, and the like. For a more detailed description of the mechanisms of action of such drugs, those of skill in the art are referred to Goodman and Gilman's "Pharmaceutical Basis of Therapeutics" Chapter 35, ninth edition, Eds. Hardman *et al.*, 1996.

In addition, local anesthetics which act by preventing the generation and conductance of the nerve impulse may also be useful as starting materials for the rational drug design discussed herein. These agents block conduction by decreasing or preventing the large transient increases in permeability of excitable membranes to  $Na^+$  that normally is produced by a slight depolarization of the membrane. This is a result of a direct interaction of the anesthetic with the voltage gated sodium channels. Exemplary anesthetic agents that may be used to block these channels include but are not limited to lidocaine, benzocaine, bupivacaine, cocaine, etidocaine, mepivacaine, promoxine, prilocaine, procaine, proparacaine, ropivacaine and tetracaine. For a more

detailed description of the mechanisms of action of such drugs those of skill in the art are referred to Goodman and Gilman's "Pharmaceutical Basis of Therapeutics" Chapter 15, ninth edition, Eds. Hardman *et al.*, 1996. Also useful in sodium channel blockade are the toxins based on tetrodotoxin and saxitoxin, two of the most potent poisons known. These agents may be used in rational drug design to produce a therapeutic that is less toxic but still an effective modulator of the sodium channel.

In addition to the above agents commonly being used in the treatment of arrhythmia, certain sodium channel blockers also are known to be effective in anti-convulsant therapy. These agents include but are not limited to phenytoin, carbamazepine, valproate, lamotrigine and topiramate. For a more detailed review of these agents and their anticonvulsant activity, those of skill in the art are referred to a recent review by Ragsdale and Avoli (1999). It is envisioned that any of these specific agents or any agents derived from these agents may be useful in various applications of the previous invention, including rational drug design, combination therapy and the like.

## **9. Assays for Determining Sodium Channel Function**

In various aspects of the present invention it may be necessary to perform a variety of assays to determine the activity of a particular voltage gated sodium channel. Such assays may measure various properties such as repulsion form TNR, cell adhesion assays, immunocytochemical assays and the like. The following section describes these assays in some detail. Of course it should be understood that the conditions described herein are merely exemplary and those of skill in the art will be able to modify or alter the conditions depending on the particular cells lines and reagents being used.

### **A. Long-term repulsion assays**

The inventors found that sodium channel  $\alpha$ ,  $\beta 1$  and  $\beta 2$  subunits, when expressed alone, are modulated specifically by TN-R to produce a repellent effect of the transfected cells away from the TN-R substrate. In investigating this effect, repulsion assays were carried out as follows.



Tissue culture 4-well or 24-well dishes are coated with methanol-solubilized nitrocellulose according to Lagenaur and Lemmon (1987). 2.5  $\mu$ l aliquots of TN-R (15 nM) and TN-C (15 nM) or GST-fusion domains of TN-R (25 nM) are applied to the nitrocellulose/poly-DL-ornithine (PO)-coated surfaces of the dishes and incubated for 2 h at 37 °C in a humidified atmosphere as described previously (Xiao *et al.*, 1996). The dishes are then washed three times with  $\text{Ca}^{2+}$  - and  $\text{Mg}^{2+}$  -free Hank's balanced salt solution (CMF-HBSS). The coating efficiency is determined as described previously (Xiao *et al.*, 1996). Substrate boundaries are marked in ink. The source of nitrocellulose (Schleicher and Schuell, catalog #401188, BA85, 0.45 $\mu$ m) is important to obtain consistent results in this assay. Parental 1610 or transfected cells are plated at a density of  $10^5$  cells/ml. After 20 h, the cells are fixed with 2.5% glutaraldehyde and stained with Coomassie blue (Sigma Chemical Co., St. Louis, Mo.) and the number of cells adhering to the extracellular matrix-coated protein areas is counted under a microscope.

#### B. Adhesion Assays

Sodium channels appear to recognize substrates such as TN-R by an initial adhesion. An exemplary adhesion assay that may be employed is described as follows. Tissue culture 4-well or 24-well dishes are coated with methanol-solubilized nitrocellulose according to Lagenaur and Lemmon (1987) and air-dried under a sterile hood. For adhesion assays, 2.5  $\mu$ l spots of different TN-R fragments or GST (each at a concentration of 25  $\mu$ M) are applied to the nitrocellulose-coated surfaces of the dishes and incubated for 2 h at 37 °C in a humidified atmosphere. The dried spots are washed with PBS and then flooded with CMF-HBSS containing 2% heat-inactivated fatty acid free BSA (Sigma) and incubated 2 h to block residual non-specific protein binding sites. The dishes are then washed with PBS and cells from the various cell lines are plated at a density of  $10^5$  cells/ml in 0.5 ml of growth medium containing 10% BSA. After 20 h of growth (5%  $\text{CO}_2$ , 37°C) cultures are fixed with CMF-HBSS containing 2.5% glutaraldehyde. For adhesion blocking assays, a mixture of EGF-L, EGF-S, and FN6-8 can be added to the culture medium. After fixation, cultures are stained with 0.5% toluidine blue in 2.5% sodium carbonate. Cells adhering to the various spots of TN-R fragments can then be photographed and counted.

#### C. Aggregation assays

Stable clones of transfected S2 cells are induced overnight in the presence of 0.7 mM CuSO<sub>4</sub> followed by aggregation for 4 h at room temperature on a rotary shaker. Cell aggregates containing at least 10 cells are then analyzed by phase contrast microscopy for homophilic aggregation or by a combination of phase contrast and fluorescent microscopy for heterophilic aggregation.

#### D. Immunocytochemical detection of ankyrin in S2 cells

Immunocytochemistry of *Drosophila* ankyrin distribution in S2 cells is performed following aggregation experiments. Cells are fixed with 2% paraformaldehyde, and permeabilized with 0.5% Triton X-100. Mouse anti-*Drosophila* ankyrin is used as the primary antibody followed by incubation with fluorescein isothiocyanate-conjugated secondary antibody. Slides are then viewed with a Bio-Rad MRC 600 confocal scanning laser microscope.

#### E. Co-immunoprecipitation of 1 and 2 subunits or subunits with neurofascin or ankyrin

Immunoprecipitations can be performed from S2 cells following protein synthesis induction but without inducing cell aggregation (cells not shaken). Cells are then pelleted and solubilized in 1.25% Triton X-100 and the soluble fraction is incubated overnight with the appropriate antibody. Protein-A-sepharose is then added and the incubation continued for 2 h. Immunoprecipitates are eluted from the Protein-A-sepharose with SDS-PAGE sample buffer and separated on 10 % SDS-PAGE gels. Proteins are then transferred to nitrocellulose and probed with the appropriate second antibody.

### 10. Diagnosing Disorders Involving $\beta$ 1A

The present inventors have determined that alterations in  $\beta$ 1A may be associated with epileptic and febrile seizures. Therefore,  $\beta$ 1A and the corresponding gene may be employed as a diagnostic or prognostic indicator of such seizures in infants. More specifically, point mutations, deletions, insertions or regulatory perturbations relating to  $\beta$ 1A may be the cause of these seizures. If it can be predicted

that an individual is predisposed to such seizures, then a prophylactic course of treatments can be designed.

#### A. Genetic Diagnosis

5 One embodiment of the instant invention comprises a method for detecting variation in the expression of  $\beta 1A$ . This may comprise determining that level of  $\beta 1A$  or determining specific alterations in the expressed product. Obviously, this sort of assay has importance in the diagnosis of related disease.

10 The biological sample can be any tissue or fluid. Various embodiments include cells of the skin, muscle, fascia, brain, prostate, breast, endometrium, lung, head & neck, pancreas, small intestine, blood cells, liver, testes, ovaries, colon, skin, stomach, esophagus, spleen, lymph node, bone marrow or kidney. Other embodiments include fluid samples such as peripheral blood, lymph fluid, ascites, serous fluid, pleural effusion, sputum, cerebrospinal fluid, lacrimal fluid, stool or urine.

15 Nucleic acid to be used in such an analysis is isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA; in another, it is poly-A RNA. Normally, the  
20 nucleic acid is amplified.

Depending on the format, the specific nucleic acid of interest is identified in the sample directly using amplification or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by visual means (*e.g.*, ethidium bromide staining of a gel).  
25 Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Affymax Technology; Bellus, 1994).

30 Following detection, one may compare the results seen in a given patient with a statistically significant reference group of normal patients and patients that have  $\beta 1A$ -related pathologies. In this way, it is possible to correlate the amount or kind of  $\beta 1A$  detected with various clinical states.

Various types of defects may be associated with  $\beta 1A$ . Thus, "alterations" should be read as including deletions, insertions, point mutations and duplications. Point mutations result in stop codons, frameshift mutations or amino acid substitutions. Somatic mutations are those occurring in non-germ line tissues. Germ line tissue mutations can occur in any tissue and are inherited. Mutations in and outside the coding region also may affect the amount of  $\beta 1A$  produced, both by altering the transcription of the gene or in destabilizing or otherwise altering the processing of either the transcript (mRNA) or protein, or altering the ratio of expression of one subunit in comparison to another.

It is contemplated that mutations in the  $\beta 1A$  gene may be identified in accordance with the present invention. A variety of different assays are contemplated in this regard, including but not limited to, fluorescent *in situ* hybridization (FISH), direct DNA sequencing, PFGE analysis, Southern or Northern blotting, single-stranded conformation analysis (SSCA), RNase protection assay, allele-specific oligonucleotide (ASO), dot blot analysis, denaturing gradient gel electrophoresis, RFLP and PCR<sup>TM</sup>-SSCP.

#### **a. Primers and Probes**

The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred. Probes are defined differently, although they may act as primers. Probes, while perhaps capable of priming, are designed to binding to the target DNA or RNA and need not be used in an amplification process.

In preferred embodiments, the probes or primers are labeled with radioactive species (<sup>32</sup>P, <sup>14</sup>C, <sup>35</sup>S, <sup>3</sup>H, or other label), with a fluorophore (rhodamine, fluorescein) or a chemilluminiscent (luciferase).

#### **b. Template Dependent Amplification Methods**

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification

methods is the polymerase chain reaction (referred to as PCR<sup>TM</sup>) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1990.

Briefly, in PCR<sup>TM</sup>, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA  
5 polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the  
10 marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR<sup>TM</sup> amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative  
15 methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641 filed December 21, 1990. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308. In LCR, two complementary probe pairs are prepared,  
20 and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR<sup>TM</sup>, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to  
25 LCR for binding probe pairs to a target sequence.

Q $\beta$  Replicase, described in PCT Application No. PCT/US87/00880, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy  
30 the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide

5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention, Walker *et al.*, (1992).

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR<sup>TM</sup>-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315). In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured

again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPO No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, PCT Application WO 89/06700, disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR<sup>TM</sup>" (Frohman, M.A., In: *PCR<sup>TM</sup> PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS*, Academic Press, N.Y., 1990; Ohara *et al.*, 1989).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention (Wu *et al.*, 1989).

#### c. Southern/Northern Blotting

Blotting techniques are well known to those of skill in the art. Southern blotting involves the use of DNA as a target, whereas Northern blotting involves the use of RNA as a target. Each provide different types of information, although cDNA blotting is analogous, in many aspects, to blotting of RNA species.

Briefly, a probe is used to target a DNA or RNA species that has been immobilized on a suitable matrix, often a nitrocellulose filter. The different species should be spatially separated to facilitate analysis. This often is accomplished by gel electrophoresis of nucleic acid species followed by "blotting" on to the filter.

Subsequently, the blotted target is incubated with a probe (usually labeled) under conditions that promote denaturation and rehybridization. Because the probe is designed to base pair with the target, the probe will bind a portion of the target sequence under renaturing conditions. Unbound probe is then removed, and detection is accomplished as described above.

#### d. Separation Methods

It normally is desirable, at one stage or another, to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See Sambrook *et al.* (1989).

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them, including column, paper, thin-layer and gas chromatography.

#### e. Detection Methods



Products may be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by a labeled probe. The techniques involved are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.* (1989). For example, chromophore or radiolabel probes or primers identify the target during or following amplification.

One example of the foregoing is described in U.S. Patent No. 5,279,721, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

In addition, the amplification products described above may be subjected to sequence analysis to identify specific kinds of variations using standard sequence analysis techniques. Within certain methods, exhaustive analysis of genes is carried out by sequence analysis using primer sets designed for optimal sequencing (Pignon *et al.*, 1994). The present invention provides methods by which any or all of these types of analyses may be used. Using the sequences disclosed herein, oligonucleotide primers may be designed to permit the amplification of sequences throughout the  $\beta$ 1A encoding nucleic acid that may then be analyzed by direct sequencing.

#### f. Kit Components

All the essential materials and reagents required for detecting and sequencing  $\beta$ 1A and variants thereof may be assembled together in a kit. This generally will

comprise preselected primers and probes. Also included may be enzymes suitable for amplifying nucleic acids, including various polymerases (RT, Taq, Sequenase<sup>TM</sup> etc.), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification. Such kits also generally will comprise, in suitable means, distinct  
5 containers for each individual reagent and enzyme as well as for each primer or probe.

**g. Design and Theoretical Considerations for Relative  
Quantitative RT-PCR<sup>TM</sup>**

Reverse transcription (RT) of RNA to cDNA followed by relative quantitative  
10 PCR<sup>TM</sup> (RT-PCR<sup>TM</sup>) can be used to determine the relative concentrations of specific mRNA species isolated from patients. By determining that the concentration of a specific mRNA species varies, it is shown that the gene encoding the specific mRNA species is differentially expressed.

In PCR<sup>TM</sup>, the number of molecules of the amplified target DNA increase by a  
15 factor approaching two with every cycle of the reaction until some reagent becomes limiting. Thereafter, the rate of amplification becomes increasingly diminished until there is no increase in the amplified target between cycles. If a graph is plotted in which the cycle number is on the X axis and the log of the concentration of the amplified target DNA is on the Y axis, a curved line of characteristic shape is formed  
20 by connecting the plotted points. Beginning with the first cycle, the slope of the line is positive and constant. This is said to be the linear portion of the curve. After a reagent becomes limiting, the slope of the line begins to decrease and eventually becomes zero. At this point the concentration of the amplified target DNA becomes asymptotic to some fixed value. This is said to be the plateau portion of the curve.

25 The concentration of the target DNA in the linear portion of the PCR<sup>TM</sup> amplification is directly proportional to the starting concentration of the target before the reaction began. By determining the concentration of the amplified products of the target DNA in PCR<sup>TM</sup> reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the  
30 specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundances of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration

of the PCR<sup>TM</sup> products and the relative mRNA abundances is only true in the linear range of the PCR<sup>TM</sup> reaction.

The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA. Therefore, the first condition that must be met before the relative abundances of a mRNA species can be determined by RT-PCR<sup>TM</sup> for a collection of RNA populations is that the concentrations of the amplified PCR<sup>TM</sup> products must be sampled when the PCR<sup>TM</sup> reactions are in the linear portion of their curves.

The second condition that must be met for an RT-PCR<sup>TM</sup> experiment to successfully determine the relative abundances of a particular mRNA species is that relative concentrations of the amplifiable cDNAs must be normalized to some independent standard. The goal of an RT-PCR<sup>TM</sup> experiment is to determine the abundance of a particular mRNA species relative to the average abundance of all mRNA species in the sample. In the experiments described below, mRNAs for  $\beta$ -actin, asparagine synthetase and lipocortin II were used as external and internal standards to which the relative abundance of other mRNAs are compared.

Most protocols for competitive PCR<sup>TM</sup> utilize internal PCR<sup>TM</sup> standards that are approximately as abundant as the target. These strategies are effective if the products of the PCR<sup>TM</sup> amplifications are sampled during their linear phases. If the products are sampled when the reactions are approaching the plateau phase, then the less abundant product becomes relatively over represented. Comparisons of relative abundances made for many different RNA samples, such as is the case when examining RNA samples for differential expression, become distorted in such a way as to make differences in relative abundances of RNAs appear less than they actually are. This is not a significant problem if the internal standard is much more abundant than the target. If the internal standard is more abundant than the target, then direct linear comparisons can be made between RNA samples.

The above discussion describes theoretical considerations for an RT-PCR<sup>TM</sup> assay for clinically derived materials. The problems inherent in clinical samples are that they are of variable quantity (making normalization problematic), and that they are of variable quality (necessitating the co-amplification of a reliable internal control,

preferably of larger size than the target). Both of these problems are overcome if the RT-PCR™ is performed as a relative quantitative RT-PCR™ with an internal standard in which the internal standard is an amplifiable cDNA fragment that is larger than the target cDNA fragment and in which the abundance of the mRNA encoding the internal standard is roughly 5-100 fold higher than the mRNA encoding the target. This assay measures relative abundance, not absolute abundance of the respective mRNA species.

Other studies may be performed using a more conventional relative quantitative RT-PCR™ assay with an external standard protocol. These assays sample the PCR™ products in the linear portion of their amplification curves. The number of PCR™ cycles that are optimal for sampling must be empirically determined for each target cDNA fragment. In addition, the reverse transcriptase products of each RNA population isolated from the various tissue samples must be carefully normalized for equal concentrations of amplifiable cDNAs. This consideration is very important since the assay measures absolute mRNA abundance. Absolute mRNA abundance can be used as a measure of differential gene expression only in normalized samples. While empirical determination of the linear range of the amplification curve and normalization of cDNA preparations are tedious and time consuming processes, the resulting RT-PCR™ assays can be superior to those derived from the relative quantitative RT-PCR™ assay with an internal standard.

One reason for this advantage is that without the internal standard/competitor, all of the reagents can be converted into a single PCR™ product in the linear range of the amplification curve, thus increasing the sensitivity of the assay. Another reason is that with only one PCR™ product, display of the product on an electrophoretic gel or another display method becomes less complex, has less background and is easier to interpret.

#### **h. Chip Technologies**

Specifically contemplated by the present inventors are chip-based DNA technologies such as those described by Hacia *et al.* (1996) and Shoemaker *et al.* (1996). Briefly, these techniques involve quantitative methods for analyzing large numbers of genes rapidly and accurately. By tagging genes with oligonucleotides or using fixed probe arrays, one can employ chip technology to segregate target molecules

as high density arrays and screen these molecules on the basis of hybridization. See also Pease *et al.* (1994); Fodor *et al.* (1991).

### B. Immunodiagnosis

5           Antibodies of the present invention can be used in characterizing the  $\beta$ 1A content of healthy and diseased tissues, through techniques such as ELISAs and Western blotting. This may provide a screen for the presence or absence of a disorder or as a predictor of future dysfunction.

10           The use of antibodies of the present invention, in an ELISA assay is contemplated. For example, anti- $\beta$ 1A antibodies are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, it is desirable to bind or coat the assay plate wells with a non-specific protein that is known to be antigenically neutral with regard to the test antisera such as bovine serum  
15           albumin (BSA), casein or solutions of powdered milk. This allows for blocking of non-specific adsorption sites on the immobilizing surface and thus reduces the background caused by non-specific binding of antigen onto the surface.

20           After binding of antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the sample to be tested in a manner conducive to immune complex (antigen/antibody) formation.

25           Following formation of specific immunocomplexes between the test sample and the bound antibody, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting same to a second antibody having specificity for  $\beta$ 1A that differs the first antibody. Appropriate conditions preferably include diluting the sample with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween<sup>®</sup>. These added agents also tend to assist in the reduction of nonspecific background. The layered antisera is then allowed to incubate for from about 2 to about 4 hr, at temperatures  
30           preferably on the order of about 25° to about 27°C. Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween<sup>®</sup>, or borate buffer.

To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the second antibody-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (*e.g.*, incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween<sup>®</sup>).

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulfonic acid (ABTS) and H<sub>2</sub>O<sub>2</sub>, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectrum spectrophotometer.

The preceding format may be altered by first binding the sample to the assay plate. Then, primary antibody is incubated with the assay plate, followed by detecting of bound primary antibody using a labeled second antibody with specificity for the primary antibody.

The antibody compositions of the present invention will find great use in immunoblot or Western blot analysis. The antibodies may be used as high-affinity primary reagents for the identification of proteins immobilized onto a solid support matrix, such as nitrocellulose, nylon or combinations thereof. In conjunction with immunoprecipitation, followed by gel electrophoresis, these may be used as a single step reagent for use in detecting antigens against which secondary reagents used in the detection of the antigen cause an adverse background. Immunologically-based detection methods for use in conjunction with Western blotting include enzymatically-, radiolabel-, or fluorescently-tagged secondary antibodies against the toxin moiety are considered to be of particular use in this regard.

#### 11. Methods for Treating $\beta$ 1A Related Disorders

The present invention also involves, in another embodiment, the treatment of disorders such as epilepsy and febrile seizures. The types of disorders that may be treated, according to the present invention, are limited only by the involvement of  $\beta$ 1A.

By involvement, it is not even a requirement that  $\beta 1A$  be mutated or abnormal - the overexpression of this protein may actually overcome dysfunction of sodium channel function within the cell. Thus, it is contemplated that a wide variety of disorders may be treated using  $\beta 1A$  based therapy, including therapies with the modulators identified in the section above, immunotherapeutics, protein therapy, gene-based therapies and combination therapies.

#### A. Immunotherapies

Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy aberrant cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, *etc.*) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells.

Immunotherapy could be used as part of a combined therapy, in conjunction with  $\beta 1A$ -targeted gene therapy. The general approach for combined therapy is discussed below. Generally, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells.

#### B. Protein Therapy

Another therapy approach is the provision, to a subject, of  $\beta 1A$  polypeptide, active fragments, synthetic peptides, mimetics or other analogs thereof. The protein may be produced by recombinant expression means or, if small enough, generated by an automated peptide synthesizer. Formulations would be selected based on the route of administration and purpose including, but not limited to, liposomal formulations and classic pharmaceutical preparations.

#### C. Genetic Based Therapies

One of the therapeutic embodiments contemplated by the present inventors is the intervention, at the molecular level, in the events involved in the malfunction of

voltage gated sodium channels. Specifically, the present inventors intend to provide, to a cell having an  $\alpha$  subunit forming the sodium channel pore, an expression construct capable of providing  $\beta 1A$  to that cell. The lengthy discussion above of expression vectors and the genetic elements are also useful in this embodiment. Particularly preferred expression vectors are viral vectors such as adenovirus, adeno-associated virus, herpesvirus, vaccinia virus and retrovirus. Also preferred is liposomally-encapsulated expression vector.

Those of skill in the art are well aware of how to apply gene delivery to *in vivo* and *ex vivo* situations. For viral vectors, one generally will prepare a viral vector stock. Depending on the kind of virus and the titer attainable, one will deliver  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$ ,  $1 \times 10^9$ ,  $1 \times 10^{10}$ ,  $1 \times 10^{11}$  or  $1 \times 10^{12}$  infectious particles to the patient. Similar figures may be extrapolated for liposomal or other non-viral formulations by comparing relative uptake efficiencies. Formulation as a pharmaceutically acceptable composition is discussed below.

Various routes are contemplated for various tissue types. The section below on routes contains an extensive list of possible routes. For practically any tissue, systemic delivery is contemplated. In addition regional delivery also may be contemplated

#### **D. Combination Therapy**

It is highly desirable to modulate the function of sodium channels in a number of clinical and therapeutic environments such as anesthesia (Duch *et al.*, 1998; French *et al.*, 1998), neuronal disorders (Ureniak and Obrenovitch, 1998), cardiac arrhythmia (Campbell and Williams, 1998; Capuchi *et al.*, 1998), epilepsy (Ragsdale and Avoli, 1998), and anticonvulsant therapy (Natsch *et al.*, 1997). Given that the present invention identifies a sodium channel  $\beta$  subunit that can be used to modulate the activity of the sodium channel, it is contemplated that protein and gene-based therapies centered on this discovery may be combined with other known modulators of sodium channel function to achieve a desirable change in sodium channel function in these settings.

To achieve such useful modulation of the sodium channel activity using the methods and compositions of the present invention, one would generally contact a "target" cell with a  $\beta 1A$  expression construct, protein, or other  $\beta 1A$  based therapeutic identified herein and at least one other agent. These compositions would be provided in a combined amount effective to alter the voltage gated sodium channel function of



the cell. This process may involve contacting the cells with the  $\beta$ 1A based therapeutic and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the  $\beta$ 1A based therapeutic and the other includes the agent.

Alternatively, the  $\beta$ 1A based treatment may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and  $\beta$ 1A based composition are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and  $\beta$ 1A based composition would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either  $\beta$ 1A or the other agent will be desired. Various combinations may be employed, where  $\beta$ 1A is "A" and the other agent is "B", as exemplified below:

A/B/A B/A/B B/B/A A/A/B B/A/A A/B/B B/B/B/A B/B/A/B

A/A/B/B A/B/A/B A/B/B/A B/B/A/A B/A/B/A B/A/A/B B/B/B/A

A/A/A/B B/A/A/A A/B/A/A A/A/B/A A/B/B/B B/A/B/B B/B/A/B

Other combinations are contemplated. Again, to achieve a therapeutic effect, both agents are delivered to a cell in a combined amount effective to alter the sodium current density in said cell.

### E. Formulations and Routes for Administration to Patients

Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions - expression vectors, virus stocks, proteins, antibodies and drugs - in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra*.

The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid

polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

5 The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or  
10 dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of  
15 the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying  
20 absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a  
25 sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered  
30 solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for

pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

5 For oral administration the polypeptides of the present invention may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic  
10 wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

15 The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can  
20 also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The  
25 formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular,  
30 subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the

proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologic Standards.

## 12. Transgenic Animals/Knockout Animals

In one embodiment of the invention, transgenic animals are produced which contain a functional transgene encoding a functional  $\beta 1A$  polypeptide or variants thereof. Transgenic animals expressing  $\beta 1A$  transgenes, recombinant cell lines derived from such animals and transgenic embryos may be useful in methods for screening for and identifying agents that induce or repress function of  $\beta 1A$ . Transgenic animals of the present invention also can be used as models for studying malfunctions of voltage gated sodium channels.

In one embodiment of the invention, a  $\beta 1A$  transgene is introduced into a non-human host to produce a transgenic animal expressing a human or murine  $\beta 1A$  gene. The transgenic animal is produced by the integration of the transgene into the genome in a manner that permits the expression of the transgene. Methods for producing transgenic animals are generally described by Wagner and Hoppe (U.S. Patent No. 4,873,191) and Brinster *et al.* 1985) and in "Manipulating the Mouse Embryo; A Laboratory Manual" 2nd edition (eds., Hogan, Beddington, Costantini and Long, Cold Spring Harbor Laboratory Press, 1994).

It may be desirable to replace the endogenous  $\beta 1A$  by homologous recombination between the transgene and the endogenous gene; or the endogenous gene may be eliminated by deletion as in the preparation of "knock-out" animals. Typically, a  $\beta 1A$  gene flanked by genomic sequences is transferred by microinjection into a fertilized egg. The microinjected eggs are implanted into a host female, and the progeny are screened for the expression of the transgene. Transgenic animals may be produced from the fertilized eggs from a number of animals including, but not limited to reptiles, amphibians, birds, mammals, and fish. Within a particularly preferred

embodiment, transgenic mice are generated which overexpress  $\beta 1A$  or express a mutant form of the polypeptide. Alternatively, the absence of a  $\beta 1A$  in "knock-out" mice permits the study of the effects that loss of  $\beta 1A$  protein has on a cell *in vivo*. Knock-out mice also provide a model for the development of  $\beta 1A$ -related disorders.

5 As noted above, transgenic animals and cell lines derived from such animals may find use in certain testing experiments. In this regard, transgenic animals and cell lines capable of expressing wild-type or mutant  $\beta 1A$  may be exposed to test substances. These test substances can be screened for the ability to enhance wild-type  $\beta 1A$  expression and or function or impair the expression or function of mutant  $\beta 1A$ .

### 13. Examples

10 The following example is included to demonstrate a preferred embodiment of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the example which follows represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute a preferred mode for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiment disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

#### EXAMPLE 1

##### Materials and Methods

20 The present example describes certain methods and materials that were employed in generating the results presented in the present invention. These are exemplary methods and those of skill in the art will understand that alternative methods may be employed in order to confirm the results obtained herein.

##### Library screening

30 A cDNA probe encoding nucleotides 345-911 of p $\beta 1.C1Aa$  (Isom *et al.*, 1992) was labeled with digoxigenin following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN) and used to screen a  $\lambda$  Express rat adrenal cDNA library prepared by Stratagene (LaJolla, CA) as previously described (Isom *et al.*, 1995a). pBK

plasmids containing cDNA inserts which hybridized strongly to the probe were rescued from the  $\lambda$  phage according to the manufacturer's instructions, confirmed by Southern blot analysis and sequenced using ThermoSequenase (Amersham Pharmacia Biotech, Piscataway, NJ). The nucleotide sequence of  $\beta$ 1A has been deposited in Gen Bank under accession number AF182949.

#### RT-PCR from rat adrenal RNA

To confirm independently that the  $\beta$ 1A transcript identified by library screening was expressed by rat adrenal gland, a region of  $\beta$ 1A from the amino terminus was amplified past the region in which the amino acid sequence changed from identity to non-identity to  $\beta$ 1, or the putative splice site, by reverse-transcriptase polymerase chain reaction (RT-PCR) using adult rat adrenal gland total RNA as template and  $\beta$ 1A3 (5'-GAAGATGAGCGCTTTGAGG-3' (SEQ ID NO:3), primer sequence common to  $\beta$ 1 and  $\beta$ 1A) and  $\beta$ 1A5 (5'-GAGAGACACAGCAAGC (SEQ ID NO:4), primer sequence unique to  $\beta$ 1A) as oligonucleotide forward and reverse primers, respectively. Rat adrenal gland cDNA was synthesized from total RNA using Superscript II (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's instructions in a total volume of 20  $\mu$ l. 2.3  $\mu$ g of total rat adrenal RNA (purified using Trizol reagent, Gibco/BRL) was used in the reaction. The PCR conditions were as follows: 1  $\mu$ l of cDNA, 0.5  $\mu$ M of each primer, 200  $\mu$ M of each dNTP (Boehringer Mannheim), 5  $\mu$ l of  $Mg^{2+}$ -free 10X PCR buffer (Perkin Elmer-Roche Molecular Systems, Branchburg, NJ), and 1.5 mM  $MgCl_2$  were mixed in a total volume of 50  $\mu$ l. Following a hot start at 94°C, 0.25  $\mu$ l of AmpliTaq DNA polymerase (Perkin Elmer) were added to the reaction tube and the amplification cycle was started. The cycling parameters were: 40 cycles of 45 sec. at 94°C, 20 sec. at 60°C, 1 min. 30 sec. at 72°C. This was followed by 10 min. at 72°C and then 4°C until the tubes were removed from the thermocycler (GeneAmp 2400, Perkin Elmer). Analysis of the PCR products on a 1% agarose gel revealed a 750 bp band. The band was excised from the gel, subcloned into pCR2.1 (Invitrogen, Carlsbad, CA), and analyzed using ThermoSequenase (Amersham Pharmacia Biotech). The sequence obtained from this PCR clone was identical to that obtained from the original  $\beta$ 1A clone plaque-purified from the adrenal cDNA library.

**Rat  $\beta 1$  gene:**

Intron 3 of the rat  $\beta 1$  gene (Makita *et al.*, 1994a) was amplified by PCR using rat genomic DNA as the template, oligonucleotides which encode  $\beta 1$  coding sequence flanking intron 3, VVDK (SEQ ID NO:5) (5'-  
5 AGATCCACCTGGAGGTGGTGGACAAGG-3' (SEQ ID NO:6)) and ANRD (SEQ ID NO:7) (5'-ACACGATGGATGCCATATCTCTGTTGG-3' (SEQ ID NO:8)) as forward and reverse primers, respectively, and the Expand Long Template PCR System (Boehringer Mannheim). All oligonucleotide primers were synthesized by Gibco/BRL. The amplification conditions were as follows: 300 ng of rat genomic DNA (Clontech Laboratories, Inc., Palo Alto, CA), 250 ng of each primer, 1mM each dNTP (Boehringer Mannheim), and 5  $\mu$ l of Expand Buffer 3 were mixed in a total reaction volume of 25  $\mu$ l. Following a hot start at 95°C, 0.5  $\mu$ l of Expand DNA polymerase were added and the amplification cycle was started. 40 cycles of the following regimen were performed: 94°C for 10 sec., 58°C for 30 sec., 68°C for 4 min. plus 20 sec. added  
10 to each successive cycle. The samples were then held at 4°C until removal from the thermocycler (GeneAmp 2400, Perkin Elmer). The 5 kb PCR product was gel-purified and sequenced directly using oligonucleotide VVDK (SEQ ID NO: 5) as the sequencing primer.

**20 RNase protection analysis:**

A plasmid containing a RNase protection probe template (pRPA-1) was constructed corresponding to nucleotides 364-533 in the  $\beta 1A$  sequence. Briefly, a 169-nucleotide Alu I/Acc I fragment was excised from pBK. $\beta 1A$  and ligated into the SmaI and Acc I sites of pBluescript (Stratagene). The resulting plasmid was then sequenced  
25 using ThermoSequenase (Amersham). To synthesize labeled cRNA, a 10  $\mu$ g aliquot of pRPA-1 was linearized with Xho I, ethanol-precipitated, resuspended in RNase-free water, and labeled with digoxigenin using the T3 MAXIscript kit (Ambion, Austin, TX) according to the manufacturer's instructions. Following a 2 hour incubation at 37°C, the reaction was incubated at 95°C for 2 minutes, chilled on ice, and then treated  
30 with RNase-free DNase (2 units) for 15 minutes at 37°C. EDTA (final concentration 30 mM) was added to stop the reaction. Free nucleotides were removed by ethanol precipitation with 0.5 M ammonium acetate and the final pellet was resuspended in 20



µl of RNase-free water. The probe (RPA-1) was quantitated by comparison of serial dilutions of the labeled probe with serial dilutions of control digoxigenin-labeled RNA supplied by Boehringer Mannheim following the manufacturer's instructions.

5 RNase protection experiments were performed using the HybSpeed RPA kit from Ambion. Briefly, 20 µg of rat embryonic day 18 brain RNA were mixed with 1 µl of digoxigenin-labeled RPA-1 probe and 30 µg of yeast tRNA in 0.5 M ammonium acetate plus 2.5 volumes of ethanol. The reaction tubes were left at -20°C for 15 minutes and the RNA was precipitated by centrifugation in a microfuge at top speed. The RNA was resuspended in 10 µl of HybSpeed hybridization buffer that had been  
10 preheated to 95°C, vortexed vigorously, and the tubes were placed at 95°C for 3 minutes. The samples were then hybridized for 10 minutes at 68°C, and digested with a mixture of RNase A and T1 (10 U/ml of RNase A and 400 U/ml RNase T1) for 30 minutes at 37°C. 150 µl of Hybspeed Inactivation/Precipitation mix were added to each reaction, and the RNA was precipitated and resuspended in 10 µl of Gel Loading  
15 Buffer 1. The reactions were electrophoresed on a 1.5 mm thick 6% acrylamide TBE denaturing gel containing 7M urea in the Mini-Protean gel format (BioRad, Hercules, CA), transferred to nylon (Boehringer Mannheim), and UV crosslinked using a Stratalinker (Stratagene, LaJolla, CA). Hybridization of the digoxigenin-labeled probe was detected with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments  
20 (1:10,000 dilution) and CSPD chemiluminescent substrate solution (Boehringer Mannheim) according to the manufacturer's instructions.

#### **Preparation of RNA and Northern blot analysis:**

25 Time-mated pregnant female Sprague-Dawley rats were anesthetized with 60 mg/kg Beuthanasia-D i.p. (Schering-Plough Animal Health Corp., Kenilworth, N.J.) and the fetuses were surgically removed. Embryonic day 9 rats were homogenized in their entirety in Trizol reagent (Gibco/BRL) to purify total RNA according to the manufacturer's instructions. Whole fetal brains were dissected at the remaining embryonic time points and total RNA was purified using Trizol reagent. RNA was also  
30 subsequently prepared from the brains and adrenal glands of the adult female rats. Postnatal rats at the indicated ages were anesthetized with Beuthanasia-D, brains were dissected and total RNA was purified with Trizol reagent. Northern blot analysis of 20

µg of each sample was performed as previously described (Isom *et al.*, 1995a) using a digoxigenin-labeled  $\beta 1A$  antisense cRNA probe encoding nucleotides 428-850 or a digoxigenin-labeled antisense cRNA probe specific to the 3' untranslated region of  $\beta 1$  (nucleotides 1053-1508 of p $\beta 1.C1Aa$ ; Isom *et al.*, 1992).

#### Construction of $\beta 1A$ expression vector:

A plasmid containing  $\beta 1$  cDNA including an in-frame amino terminal hemagglutinin (HA) epitope tag was obtained as a generous gift from the laboratory of R.A. Maue at Dartmouth University (Shah *et al.*, 1996). This construct has been shown to express functional  $\beta 1$  subunits in *Xenopus* oocytes. The HA-tagged  $\beta 1$  cDNA was recloned into the Eco RI and Not I sites of the mammalian expression vector pCIneo (Promega, Madison, WI) to create pCI. $\beta 1$ -HA. pCI. $\beta 1$ -HA was subsequently digested with Acc I and Not I and agarose gel-purified to remove the 3' end of  $\beta 1$ . The Acc I restriction endonuclease site is common to  $\beta 1$  and  $\beta 1A$ . pBK. $\beta 1A$  cDNA was digested with Acc I and Not I and gel-purified. The 3' end of  $\beta 1A$  was then ligated into Acc I/Not I-digested pCI. $\beta 1$ -HA to create pCI. $\beta 1A$ -HA. The junctions were then sequenced to confirm that the segments of  $\beta 1$  and  $\beta 1A$  were successfully ligated in frame.

#### Transfection of SNaIIA cells with HA-tagged $\beta 1A$ :

SNaIIA cells were transfected with pCI. $\beta 1A$ -HA using DOTAP as previously described (Isom *et al.*, 1995b). Because SNaIIA cells are resistant to G418 as a result of the original transfection of the  $\alpha$ IIA subunit, pCI. $\beta 1A$ -HA was cotransfected with pSV2\*Hyg to confer resistance to the antibiotic hygromycin. Drug selection with hygromycin (400 µg/ml) required approximately 1 week. Clonal cell lines were selected, analyzed by Northern blot, and expanded as previously described (Isom *et al.*, 1995b).

#### [ $^3H$ ] Saxitoxin binding analysis:

Whole cell saturation binding analysis of SNaIIA and SNaIIA- $\beta 1A$  cells was performed as previously described (Isom *et al.*, 1995b) over a concentration range of

0.1 to 10 nM [ $^3\text{H}$ ]STX with the addition of 10  $\mu\text{M}$  unlabeled tetrodotoxin (TTX; Calbiochem, San Diego, CA) to assess non-specific binding.  $^3\text{H}$ -Saxitoxin ( $^3\text{H}$ -STX, 28 Ci/mmol) was obtained from Amersham. Binding data were normalized to protein concentration using the BCA Protein Assay kit (Pierce, Rockford, IL). Saturation binding data were analyzed by non-linear regression using Prism (GraphPad Software, LaJolla, CA) to obtain  $K_D$  and  $B_{\text{max}}$  values.

#### Antibodies:

A multiple antigenic peptide (MAP) with amino acid sequence RWRDRWKEGDRLVSHRGQ (SEQ ID NO: 9), encoded by nucleotides 160 through 177 of  $\beta 1A$ , was synthesized by the Protein and Carbohydrate Structure Facility at the University of Michigan. Rabbit polyclonal antibodies were subsequently generated in 2 separate animals and tested by ELISA against the  $\beta 1A$ -MAP to determine the antibody titer (Research Genetics, Inc., Huntsville, AL). Serum used for the Western blots in this study came from animal #86051.

#### Western blot analysis of $\beta 1A$ protein expression:

Adult female Sprague-Dawley rats were sacrificed by decapitation. Brain, spinal cord, heart, skeletal muscle, and adrenal gland tissues were immediately removed, minced, and briefly stored on ice. SNa $\beta 1A$ -16 cell line cells were washed with PBS and scraped into 50 ml conical tubes. Membranes were prepared as described previously (Isom *et al.*, 1995) and the final pellets were resuspended in 50 mM Tris pH 8, 10 mM EGTA containing Complete-Mini protease inhibitor tablets according to the manufacturer's instructions (Boehringer Mannheim). The total protein in each membrane preparation was quantitated with the BCA Protein Assay Kit (Pierce) using BSA as the standard. 250  $\mu\text{g}$  of each membrane preparation were separated by SDS-PAGE as previously described (Isom *et al.*, 1995), transferred to nitrocellulose (HyBond ECL, Amersham), and stained with Ponceau-S prior to immunodetection. Western blot analysis was performed as follows: the blot was washed for 10 minutes in TBS-T (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) at room temperature and then blocked for 1 hour in 5% non-fat dry milk in TBS-T at room temperature. Primary anti- $\beta 1A$  antibody (1:750 dilution) was applied in blocking solution for 30 minutes at

room temperature. The blot was then washed 5 times for 15 minutes each in TBS-T. Secondary antibody (horse radish peroxidase-conjugated goat anti-rabbit IgG, ICN) diluted to 1:100,000 in blocking solution was applied for 30 minutes at room temperature. The blot was then washed 5 times for 15 minutes each in TBS-T.

5 SuperSignal WestFemto chemiluminescent substrate solution (Pierce) was applied according to the manufacturer's instructions, the blot was placed between plastic sheet protectors, and exposed to Hyperfilm-ECL (Amersham) for the indicated times (typically 10 to 30 seconds) at room temperature.

#### 10 Immunohistochemical Analysis of $\beta 1A$ expression:

Rat tissues were routinely fixed in 10% neutral buffered formalin, processed, embedded in paraffin blocks, sectioned onto slides. The rabbit anti-rat  $\beta A1$  antibody was used at titer of 1:900 for the human tissues and 1:600 for the rat tissues. Briefly, slides were incubated (all incubations for 30 min. at room tem) with primary antibodies  
15 then incubated with specific biotin conjugated secondary antibodies (Vector Labs, Burlingham, CA) which were then detected using the ABC-horseradish peroxidase system (Vector Labs, Burlingham, CA) followed by 3'-diaminobenzidine (Biomeda, Foster City, CA) as the chromogen, stained in Mayer's hematoxylin and coverslipped with Permount (Fisher, Pittsburgh, PA).

#### 20 Electrophysiological analysis:

Electrophysiological recordings on SNaIIA and SNaIIA $\beta 1A$  cells were performed by the patch clamp technique in the whole cell configuration (Hamill *et al.*, 1981), using an Axopatch 200B patch clamp amplifier and pCLAMP software (Axon Instruments). Data were filtered at 5 kHz and digitally sampled at 50 kHz. Series  
25 resistance was compensated 60-80%. Capacitive transients, elicited by voltage steps, were partially canceled using the internal clamp circuitry. Additional subtraction of transients and leak currents was obtained using the P/4 procedure (Armstrong and Bezanilla 1977). For whole cell recordings, recording pipettes were filled with 105 mM CsF, 10 mM CsCl, 10 mM NaCl, 10 mM EGTA, 10 mM HEPES, pH 7.4 with CsOH.  
30 Pipette resistances were 1-3 M $\Omega$ . The bath solution consisted of 130 mM NaCl, 4 mM KCl, 1.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM Glucose, 10 mM HEPES, pH 7.4 with NaOH. As has been previously described (Isom *et al.*, 1995), the voltage-dependence of

sodium current activation and inactivation progressively shifted to more negative potentials over the first few minutes of experiments with fluoride-based intracellular solutions. Thus, all experiments were begun 10 min after break in, at which point the shifts in channel gating had stabilized.

5 For each cell, the voltage-dependence of current activation and steady state inactivation was examined. Activation was assessed by applying test pulses to potentials from -50 to +70 mV in 5 mV steps, following a 100 ms prepulse to -100 mV. Peak current amplitude ( $I_{\text{peak}}$ ) was measured at each test potential, and converted to conductance ( $g$ ) according to  $g = I_{\text{peak}}/(V_{\text{rev}} - V_{\text{test}})$ , in which  $V_{\text{test}}$  is the test potential and  
10  $V_{\text{rev}}$  is the current reversal potential, determined by linear extrapolation of the straight line portion of the falling phase of the current-voltage relationship. The conductance values were normalized with respect to the maximal conductance, plotted as a function of  $V_{\text{test}}$ , and fit with the Boltzman equation:  $1/(1 + \exp((V_{\text{test}} - V_{1/2})/k))$ , in which  $V_{1/2}$  is the midpoint of the curve and  $k$  is a slope factor. Steady state inactivation was examined  
15 by applying 100 ms long prepulses to potentials ranging from -100 to -10, in 5 mV steps, followed by a test pulse to 0 mV. The peak amplitude of currents evoked by the test pulses were normalized with respect to the largest currents, plotted as a function of prepulse potential and fit with the Boltzman equation.

#### 20 Spinal Nerve Ligation Surgery:

Spinal nerve ligation (SNL) was performed as described by Kim and Chung (1992). Briefly, male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing approximately 200g were anesthetized with isoflurane. The spinal nerve at the level of L5 or L6 was exposed through an incision left of the dorsal midline and tightly ligated with 6-0 silk.  
25 The spinal nerve was visualized without being ligated in the sham-operated animals. Naïve animals did not undergo surgery. Mechanical allodynia was assessed according the methods of Chaplan et al. (1994) at various times (2 days, 14 days, and 8 weeks) after surgery. SNL animals were included in the study if the ipsilateral paw consistently responded with a paw withdrawal threshold of less than 4 grams of  
30 pressure. The naïve animals and sham-operated animals did not demonstrate allodynia to the mechanical stimulus, which was greater than 15 grams of force.

**Tissue Preparation of SNL-treated subjects:**

At 2 days, 14 days and 8 weeks after surgery, sham-operated and SNL animals (n=3-5) as well as the naïve animals (n=3) were sacrificed by CO<sub>2</sub> asphyxiation. Animals were transcardially perfused with 4% paraformaldehyde, and the dorsal root ganglia (DRG) tissue was removed from the ipsilateral and contralateral sides. These tissues were processed for paraffin embedding, serially sectioned at 5µm, mounted onto Superfrost-Plus slides (Fisher, Pittsburgh, PA), and then processed for immunohistochemistry.

**Quantitation of immunohistochemical staining in SNL-treated subjects:**

Tissue sections were processed simultaneously to minimize potential staining variability. Ipsilateral and contralateral DRG neurons were characterized as nociceptive if the diameter was < 25 µm; all others were designated as sensory (Waxman, et al., 1999). An average of 30-40 neurons were quantified for immunoreactivity and scored as follows: 0.0 for no staining, 1.0 for weak staining, 2.0 for moderate immunoreactivity, and 3.0 for intense immunoreactivity. These data were then averaged per animal (n=3-5) per group for the intensity of β1A and β1 immunolabeling.

The morphologic patterns of the β1A and β1 immunoreactivity in the nociceptive and sensory DRG neurons were also characterized as 1) homogeneous for a diffuse labeling pattern; 2) punctate for several clumpy, intracellular, Nissle-like aggregates of staining; and 3) membrane for peripheral labeling located predominantly inside the cell, along the cell membrane. Data are presented as a percentage of labeling pattern observed per DRG per group.

**EXAMPLE 2****Molecular cloning and analysis of β1A**

A rat adrenal gland cDNA library prepared in the λExpress vector was screened with a digoxigenin-labeled cDNA probe encoding nucleotides 345-911 of pβ1.C1Aa (Isom *et al.*, 1992). A clone encoding a protein with a 5' region of identity to β1 and a novel 3' region was identified by DNA sequencing. The identity of this clone was then confirmed independently by reverse transcriptase polymerase chain reaction (RT-PCR) from rat adrenal cDNA using the oligonucleotides β1A3 and β1A5 followed by DNA

sequencing, as described in Experimental Procedures. This clone, designated  $\beta 1A$ , encoded a novel 253 amino acid protein of 29,055 daltons (predicted molecular mass of the mature protein with the signal sequence removed) which contains a predicted amino terminal region of identity to  $\beta 1$ , residues met (-1) through lys (129), followed by a novel carboxy terminal region (FIG. 1A and FIG. 1B). Hydrophobicity analysis of the novel, carboxy-terminal region revealed an apparent 66-amino acid extension of the extracellular region of  $\beta 1$  followed by a 19-amino acid transmembrane domain and short, 39-amino acid intracellular carboxy-terminus (FIG. 1A and FIG. 1C). The novel 3' region of  $\beta 1A$  is structurally homologous to  $\beta 1$  in that it predicts a transmembrane domain and short intracellular region, yet it contains little to no homology at the amino acid level (FIG. 1B). Interestingly, the amino terminal region common to  $\beta 1$  and  $\beta 1A$  contains the extracellular immunoglobulin fold.  $\beta 1A$  can thus be characterized as a cell adhesion molecule like  $\beta 1$  and  $\beta 2$ .

Analysis of the novel 3' region of  $\beta 1A$  by BLAST-P search of the Swissprot database revealed a 55-residue region of  $\beta 1A$  with 32% identity to an extracellular LDL-receptor class A domain of human low-density lipoprotein receptor-related protein 2 (LRP2), also called megalin or glycoprotein 330 (FIG. 1D; Kounnas *et al.*, 1995; Gliemann, 1998; Saito *et al.*, 1994; Korenberg *et al.*, 1994). This region of homology in  $\beta 1A$  is predicted to be located extracellularly, just proximal to the plasma membrane followed by the transmembrane region itself. LRP2 has been shown to be a cysteine-rich type I membrane protein that forms a multimeric complex with receptor-associated protein (RAP). LRP2 binds clusterin with high affinity and is localized to clathrin-coated pits, suggesting that it may be an endocytic receptor. Interestingly, LRP2 interacts with extracellular matrix components, similar to sodium channel  $\beta 1$  and  $\beta 2$  subunits. The BLAST-P search also revealed a 63-residue region of  $\beta 1A$  with 26% identity to tensin, a protein that has been implicated as the anchor for actin filaments at focal adhesions and is thought to act as a link between the cytoskeleton and signal transduction proteins (Weigt *et al.*, 1992). The region of homology to  $\beta 1A$  is located in the insertin domain of tensin. This domain has been shown to permit polymerization of actin filaments.

### EXAMPLE 3

**$\beta$ 1A is encoded by a retained intron in the  $\beta$ 1 gene**

The genomic organization of the human sodium channel  $\beta$ 1 subunit gene has been reported previously (Makita *et al.*, 1994). The  $\beta$ 1 gene contains 5 introns and 6 exons. The  $\beta$ 1A cDNA is the result of retention of I3, creating a novel 3' end. It was determined that the site of divergence between the  $\beta$ 1 and  $\beta$ 1A cDNAs was precisely located at the boundary between exon 3 and intron 3 of the  $\beta$ 1 gene. Furthermore, a consensus sequence for exon-intron boundaries in genomic DNA (Mount, 1982) was readily identified at this location. Sequencing of a PCR-generated product from rat genomic DNA of intron 3 (approximately 5 kb) showed that the sequence of  $\beta$ 1A beyond the amino acid sequence VVDK was indeed that of intron 3. RNase protection experiments were performed using a probe that spanned the exon 3-intron 3 boundary in the rat sequence; this 169 nucleotide probe was fully protected by rat embryonic day 18 brain mRNA. Thus, it is proposed that the novel extracellular, transmembrane, and carboxy terminal domains of  $\beta$ 1A are encoded by alternative splicing of a retained intron within the  $\beta$ 1 gene that includes an in-frame termination codon. These data are in agreement with the previously reported observation that  $\beta$ 1 is represented only once in the rat and human genomes (Makita *et al.*, 1994; Tong *et al.*, 1993).

**EXAMPLE 4** **$\beta$ 1A mRNA is expressed in embryonic brain and adult adrenal gland**

A comparison of the developmental time courses of  $\beta$ 1A and  $\beta$ 1 mRNA expression in developing rat brain was determined using specific, non-cross-hybridizing antisense cRNA probes for  $\beta$ 1A and  $\beta$ 1, respectively. Time-mated pregnant female Sprague-Dawley rats were anesthetized with 60 mg/kg Beuthanasia-D i.p., and the fetuses were surgically removed. Embryonic day 9 fetuses were homogenized in their entirety in Trizol reagent to purify total RNA according to the manufacturer's instructions. Whole fetal brains were dissected at the remaining embryonic time points, and total RNA was purified using Trizol reagent. RNA was also prepared from the brains and adrenal glands of the adult female rats. Postnatal rats at the indicated ages were anesthetized with Beuthanasia-D, brains were dissected and total RNA was purified with Trizol reagent. Northern blot analysis of 20  $\mu$ g of each



sample was performed as previously described (Isom *et al.*, 1995a) using a digoxigenin-labeled  $\beta 1A$  antisense RNA probe encoding nucleotides 428-850 or a digoxigenin-labeled antisense RNA probe specific to the 3' untranslated region of  $\beta 1$  (nucleotides 1053-1508 of p $\beta 1.C1Aa$ ; Isom *et al.*, 1992). Interestingly, the expression time course of  $\beta 1A$  parallels that of the 26 kDa  $\beta 1$ -immunoreactive band described previously (McHugh-Sutkowski and Catterall, 1990) and complements the expression pattern of  $\beta 1$  (Patton *et al.*, 1994). Thus,  $\beta 1A$  is expressed early in development and disappears after birth. In contrast,  $\beta 1$  expression is not detectable during embryonic brain development and becomes detectable as  $\beta 1A$  mRNA expression is decreased.

### EXAMPLE 5

#### Analysis of $\beta 1A$ protein expression

To determine whether alternative splicing of the  $\beta 1$  gene resulted in expression of a novel protein, a polyclonal antibody was generated against a MAP peptide containing the amino acid sequence RWRDRWKEGDRLVSHRGQ (SEQ ID NO:9) encoded by the retained portion of intron 3 found in the  $\beta 1A$  cDNA clone. Brain, spinal cord, heart, skeletal muscle, and adrenal gland membranes were prepared as described previously (Isom *et al.* 1995). Total protein in each membrane preparation was quantitated with the BCA Protein Assay Kit. 250  $\mu$ g of each membrane preparation were separated by SDS-PAGE as previously described (Isom *et al.*, 1995) and transferred to nitrocellulose. Western blot analysis consisted of the following steps, all at room temperature: the blot was first washed for 10 minutes in TBS-T (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and then blocked for 1 hour in 5% non-fat dry milk in TBS-T at room temperature. Primary anti- $\beta 1A$  antibody (1:750 dilution) was applied in blocking solution for 30 minutes. After washing the blot 5 times for 15 minutes each in TBS-T, secondary antibody (horse radish peroxidase-conjugated goat anti-rabbit IgG, ICN) diluted to 1:100,000 was applied in blocking solution for 30 minutes. After washing the blot as before, SuperSignal WestFemto chemiluminescent substrate solution was applied according to the manufacturer's instructions, the blot was placed between plastic sheet protectors, and exposed to Hyperfilm-ECL for 10 seconds at room temperature.  $\beta 1A$ -immunoreactive bands that migrated at approximately 50 kDa were observed in heart, skeletal muscle, and adrenal

gland, but were not detected in brain or spinal cord. An immunoreactive doublet was observed in adrenal gland. The absence of immunoreactive  $\beta 1A$  protein bands in the CNS tissues brain and spinal cord is consistent with the Northern blot results.

## EXAMPLE 6

### Immunocytochemical Analysis of $\beta 1A$ Expression

Various central and peripheral neuronal populations contained  $\beta 1A$  when analyzed immunocytochemically with anti- $\beta 1A$  primary and horse-radish peroxidase conjugated secondary antibodies. These studies demonstrate that  $\beta 1A$  was found in most but not all Purkinje cells in the cerebellum. In addition, some  $\beta 1A$  positive neurons in the dentate nucleus of the cerebellum were observed.  $\beta 1A$  was absent from the granular layer and the molecular layer of the cerebellum. A distinct population of pyramidal neurons of the cerebral cortex contained  $\beta 1A$ , while glial cell populations remained negative. Spinal cord also contained several distinct populations of  $\beta 1A$  containing neurons. The  $\beta 1A$  protein was also localized to a small population of motor neurons, while  $\beta 1A$ -expressing neurons were also observed in laminae II-V of the dorsal horn. All neuronal cell types of the dorsal root ganglia contained  $\beta 1A$ , while fiber tracks and glial cells did not stain.

In addition, non-neuronal cells were also found to express  $\beta 1A$ . These include endomysium membranes of individual muscle fibers in rat atria, other areas of the rat heart such as ventricles, lung alveoli and some bronchus columnar epithelial cells expressed  $\beta 1A$  protein.

## EXAMPLE 7

### Mammalian Cell Expression

To investigate the functional role of  $\beta 1A$  a hemagglutinin (HA) epitope-tagged version of the  $\beta 1A$  cDNA was constructed. An epitope tag was included for potential use in the event that the polyclonal antibody production was unsuccessful. However, the HA tag experiments was not necessary as this approach was successful in raising an anti- $\beta 1A$  antibody. Stably transfected cells lines expressing  $\beta 1A$  were created in the previously-characterized SNaIIA cell line. SNaIIA cells are a stable line expressing type IIA sodium channel  $\alpha$  subunits in Chinese hamster lung (CHL) cells (Isom *et al.*,

1995). Because SNaIIA cells are G418-resistant, pcDNA3- $\beta$ 1A was co-transfected with pSV2\*Hyg in a 10:1 ratio (pcDNA3- $\beta$ 1A: pSV2\*Hyg) so that transfected clones could be selected with the antibiotic hygromycin. A number of hygromycin-resistant colonies were analyzed by Northern blot for  $\beta$ 1A mRNA expression. Positive clones were expanded and analyzed further by [ $^3$ H]-STX binding. Western blot analysis of one of these cell lines, SNaIIA $\beta$ 1A-16, identified an immunoreactive band of approximately 45 kDa.

[ $^3$ H]-STX binding analysis revealed a significant increase in the expression levels of functional sodium channels at the plasma membrane of SNaIIA $\beta$ 1A-16 cells as compared to the parent line, SNaIIA (Table 2). Non-linear regression analysis of saturation binding showed a 4.4-fold increase in  $B_{\max}$  as compared to SNaIIA with no significant change in the  $K_D$  (0.8 nM for SNaIIA vs. 0.9 nM for SNaIIA $\beta$ 1A-16).

**Table 2.  $^3\text{H}$ -Saxitoxin binding analysis of  $\alpha\text{IIA}$ - and  $\alpha\text{IIA}/\beta\text{1A}$ -expressing cell lines.** Whole cell saturation binding analysis of SNAIIA and SNaIIA- $\beta\text{1A}$  cells was performed as previously described (Isom *et al.*, 1995b) over a concentration range of 0.1 to 10 nM [ $^3\text{H}$ ]STX with the addition of 10  $\mu\text{M}$  unlabeled tetrodotoxin to assess non-specific binding.  $^3\text{H}$ -Saxitoxin ( $^3\text{H}$ -STX, 28 Ci/mmol) was obtained from Amersham. Binding data were normalized to protein concentration using the BCA Protein Assay kit. Saturation binding data were analyzed by non-linear regression using Prism to obtain  $K_D$  and  $B_{\text{max}}$  values.

| cell line                    | $B_{\text{max}}$ +/- SEM | $K_D$ +/- SEM    |
|------------------------------|--------------------------|------------------|
| SNaIIA                       | 7.9 +/- 1.6 fmol/mg      | 0.81 +/- 0.53 nM |
| SNaIIA- $\beta\text{1A}$ -16 | 35.1 +/- 8.8 fmol/mg     | 0.92 +/- 0.89 nM |

These results are similar to a previous study showing that coexpression of  $\alpha$ IIA and  $\beta$ 1 resulted in a 2- to 4-fold increase in the level of [ $^3$ H]-STX binding compared to cells expressing  $\alpha$ IIA alone (Isom, *et al.*, 1995). The  $K_D$  values obtained in the present study were very similar to those reported values as well. These data suggest that a function common to  $\beta$ 1 and  $\beta$ 1-A is to increase the level of sodium channel expression at the plasma membrane. The inventors suggest that  $\beta$ 1 and  $\beta$ 1-A may stabilize the conformation of channels such that they become more resistant to degradation and/or target newly synthesized channels to the plasma membrane from intracellular stores. Because  $\beta$ 1 and  $\beta$ 1A contain a common cell adhesion molecule domain, it is proposed that the extracellular Ig loop may be necessary for this function.

To determine whether coexpression of  $\beta$ 1A subunits affected the functional properties of type IIA sodium channels in CHL cells, whole cell electrophysiological recording was used to compare sodium currents in the parent SNaIIA cell line and in three different SNaIIA $\beta$ 1A cell lines. FIG. 2A shows currents, elicited by depolarizations to varying test potentials, recorded in a typical SNaIIA cell and a typical SNaIIA $\beta$ 1A cell. As is evident from these traces, coexpression of  $\beta$ 1A did not dramatically alter the properties of voltage-activated sodium currents. Nevertheless, currents in  $\beta$ 1A-expressing cell lines were subtly different from currents in SNaIIA cells. For example, mean steady state inactivation curves for SNaIIA $\beta$ 1A cell lines were shifted to more positive potentials than the mean inactivation curve for SNaIIA cells (FIG. 2B and FIG. 2D). Although this difference was quite small, it was observed in all three SNaIIA $\beta$ 1A cell lines, and was statistically significant in two of the three  $\beta$ 1A-containing lines (SNaIIA $\beta$ 1A-7:  $p = 0.037$ ; SNaIIA $\beta$ 1A-8:  $p = 0.024$ ). Thus, one effect of  $\beta$ 1A association with  $\alpha$ IIA may be a small positive shift in the voltage-dependence of steady state inactivation. For activation, mean voltage-conductance curves for two of the three  $\beta$ 1A expressing lines were statistically indistinguishable from SNaIIA (FIG. 2B, FIG. 2D); however, for SNaIIA $\beta$ 1A-16, the voltage-dependence of activation was shifted to a significantly more negative membrane potential ( $p = 0.001$ ). Thus, data from one of the three  $\beta$ 1A cell lines suggests that  $\beta$ 1A may also alter sodium channel activation.

To determine whether  $\beta 1A$  affected the time course of macroscopic sodium currents, the decaying phase of whole cell currents, elicited over a range of test potentials, was fit with single exponential functions (FIG. 2C). For SNaIIA, the inactivation time constants determined from these fits were progressively shorter at progressively more positive test potentials, approaching a minimum of approximately 0.5 ms at the most positive test potentials examined. For two of the three SNaIIA $\beta 1A$  cell lines, the rate of current inactivation was virtually identical to SNaIIA (FIG. 2C). However, for SNaIIA $\beta 1A$ -16, inactivation was faster at all test potentials examined (FIG. 2C). Because sodium channel inactivation is coupled to activation (Aldrich *et al.*, 1983; Bezanilla, 1977), it is likely that the faster decay rate for SNaIIA $\beta 1A$ -16 currents were, at least in part, secondary to the negative shift in activation that was observed in this cell line. In addition, it is also possible that, similar to  $\beta 1$ ,  $\beta 1A$  subunits have direct effects on the rate of sodium channel inactivation.

The most dramatic effect of  $\beta 1A$ , detected electrophysiologically, was a large increase in the amplitudes of macroscopic sodium currents. FIG. 3A shows amplitudes of currents evoked by depolarization to +10 mV in SNaIIA and SNaIIA $\beta 1A$  cell lines, converted to current densities to factor out any differences in cell surface area. Current densities for SNaIIA cells were 6.9 pA/pF, whereas current densities were approximately 2.5 times greater for the three SNaIIA $\beta 1A$  cell lines (FIG. 3A). This difference reflected two distinct effects of  $\beta 1A$  on sodium channel expression. First,  $\beta 1A$  greatly increased the proportion of cells with measurable whole cell sodium currents. This effect is illustrated in FIG. 3B, which plots the number of SNaIIA (black bars) or SNaIIA $\beta 1A$  (white bars) cells with peak currents within different amplitude ranges. For SNaIIA, this amplitude-frequency distribution was bimodal. In 40% of the cells (16 out of 40) currents were indistinguishable from the small inward currents recorded in untransfected CHL cells (*i.e.* < 100 pA). In the remaining 60% of the cells, currents, ranged from 500 pA to 5 nA, and thus were clearly due to expression of cloned type IIA channels. In contrast, all SNaIIA $\beta 1A$  cells expressed large sodium currents (FIG. 3A). The frequency histogram for SNaIIA $\beta 1A$  followed a normal distribution with a modal current range of 2-3 nA.

To determine whether the lower mean current density of SNaIIA cells was solely due to its large proportion of low expressing cells, the mean current density for

SNaIIA, excluding these low expressers was recalculated. Eliminating low expressing cells increased the mean current density for SNaIIA cells from 6.9 pA/pF to 11.6 pA/pF; however, this value was still significantly smaller than the mean current density of cells expressing  $\beta 1A$  ( $p = 0.014$ ). Thus, even when comparing only those cells that expressed measurable sodium currents,  $\beta 1A$  still increased the density of functional sodium channels on the cell surface.

### EXAMPLE 8

#### $\beta 1A$ expression in neuropathic pain

To better understand the relationship it may have in neuropathic pain,  $\beta 1A$  subunit expression and localization was assessed in neural tissues after spinal nerve injury. In this example, spinal nerve ligation in rats was used to simulate injury.

Patterns of  $\beta 1$  and  $\beta 1A$  subunit expression levels differed from each other in the SNL-treated animals. Staining intensity by anti- $\beta 1A$  subunit antibodies in the DRG exhibited a post-surgical time-dependent increase. Labeling intensity in nociceptive neurons was slightly elevated at two days post-surgery, and labeling in both nociceptive and sensory populations was enhanced at two weeks. Importantly, and in parallel with the continued expression of allodynic behavior,  $\beta 1A$  labeling remained elevated at eight weeks post-surgery. Labeling increases were more prominent in the L5 than in the L4 regions, and in the ipsilateral than the contralateral DRG neurons.

While  $\beta 1A$  levels continued to increase in SNL animals,  $\beta 1$  subunit expression levels varied. DRG labeling by anti- $\beta 1$  subunit antibodies was more intense at the L5 than at the L4 regions in naïve animals. Post surgically, the labeling intensity either was unchanged or decreased slightly at day 2. At two-weeks post-surgery, a slight increase in labeling was observed at the L5 and a more pronounced increase at the L4 level. At the eight week post-surgical time point, labeling in the L5 DRG was further elevated, whereas the labeling in the L4 DRG was similar to that observed at the two week time point. Thus, the  $\beta 1$  subunit exhibits a biphasic post-surgical expression pattern.

Immunohistochemical experiments indicated that the subcellular distribution of the  $\beta 1$  subunit was uniformly diffuse or homogenous, whereas several distinct labeling patterns were observed for the  $\beta 1A$  subunit in both nociceptive and sensory neurons

(Table 3). In naïve or sham operated rats,  $\beta 1A$  labeling was predominantly homogeneous in both nociceptive and sensory neurons of the L5 region. At 14 days after SNL, however, the  $\beta 1A$  expression pattern was markedly altered. In nociceptive neurons, the dominant labeling was proximal to the membrane, one quarter was punctate, and less than one quarter of the labeling remained homogeneously distributed. In sensory neurons in the ipsilateral DRG to the SNL as well as in both nociceptive and sensory neurons in the contralateral DRG, similar  $\beta 1A$  expression patterns were observed. In these populations, 60-75% of the labeling remained diffuse, whereas up to 28% of the  $\beta 1A$  expression localized proximal to the membrane in contralateral nociceptive neurons, or was punctate in ipsilateral and contralateral sensory neurons.



**Table 3. Relative distribution of the morphologic patterns of  $\beta 1A$  and  $\beta 1$** 

**expression in L5 DRG.** Immunoperoxidase staining of 5  $\mu$ m sections with polyclonal antibodies specific for either the  $\beta 1$  or  $\beta 1A$  subunit of the sodium channel. Labeling patterns were "membrane" if the signal was relegated to the cell plasma membrane, "punctate" if the signal was intracellular aggregates, and "homogenous" if staining was diffuse. Percentages of labeling patterns are presented as observed per DRG per group. Treatment groups: naïve, untreated; sham, surgery exposing, but not disturbing, the spinal nerve; SNL, spinal nerve ligation surgery-treated animals.

| Antibody   | Side          | Neuron Class | Labeling patterns (%) | Naïve | Sham (14d) | SNL (14d) |
|------------|---------------|--------------|-----------------------|-------|------------|-----------|
| $\beta 1A$ | Ipsilateral   | Nociceptive  | Membrane              |       | 15         | 51        |
|            |               |              | Punctate              | 23    | 16         | 26        |
|            |               |              | Homogeneous           | 77    | 69         | 23        |
|            |               | Sensory      | Membrane              |       |            | 11        |
|            |               |              | Punctate              | 18    | 11         | 18        |
|            |               |              | Homogeneous           | 82    | 89         | 71        |
|            | Contralateral | Nociceptive  | Membrane              |       |            | 28        |
|            |               |              | Punctate              |       |            | 14        |
|            |               |              | Homogeneous           | 100   | 100        | 58        |
|            |               | Sensory      | Membrane              |       |            | 4         |
|            |               |              | Punctate              |       |            | 20        |
|            |               |              | Homogeneous           | 100   | 100        | 76        |
| $\beta 1$  | Ipsilateral   | Sensory      | Membrane              |       |            |           |
|            |               |              | Punctate              |       |            |           |
|            |               |              | Homogeneous           | 100   | 100        | 100       |
|            |               | Nociceptive  | Membrane              |       |            |           |
|            |               |              | Punctate              |       |            |           |
|            |               |              | Homogeneous           | 100   | 100        | 100       |
|            | Contralateral | Sensory      | Membrane              |       |            |           |
|            |               |              | Punctate              |       |            |           |
|            |               |              | Homogeneous           | 100   | 100        | 100       |
|            |               | Nociceptive  | Membrane              |       |            |           |
|            |               |              | Punctate              |       |            |           |
|            |               |              | Homogeneous           | 100   | 100        | 100       |

The SNL-induced increase in  $\beta 1A$  labeling intensity, coupled with the dramatic post-surgical alteration of the subunit's subcellular distribution, correlates with a role for the  $\beta 1A$  subunit in the development and maintenance of neuropathic pain observed in this model.

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## EXAMPLE 9

### Production of Mutants

The present Example briefly describes the generation of specific mutants contemplated by the present inventors. Such mutants will yield specific information about the function and properties of particular regions of the  $\beta 1A$  protein. The inventors propose that the transmembrane and/or COOH-terminal intracellular domains of the  $\beta$  subunits are responsible for transducing a signal from the extracellular site of TN-R interaction to cytoskeletal or signaling molecules inside the cell. Truncation mutants that eliminate the intracellular COOH-terminal domains of  $\beta 1$  and  $\beta 2$  can thus be made and tested in the cell migration assays for adhesion and repulsion to determine the role of these domains in transducing signals from TN-R to the intracellular environment.

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Introduction of COOH-terminal signal peptides that eliminate the transmembrane domain and add glycosylphosphatidylinositol (GPI) lipid anchors to the  $\beta 1A$  or other  $\beta$  subunits will be fused with the extracellular domains of the  $\beta$  subunits.

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The inventors also will construct expression vectors that contain point mutations of amino acids in  $\beta 1A$  that are predicted to be located in the extracellular Ig fold. With these constructs it will be possible to determine whether the Ig fold contains the site for TN-R interaction. In addition these data will provide information as to which residues in the Ig fold are critical for  $\alpha\beta 1A$  interaction. Correlating the amino acid sequences of  $\beta 1A$ ,  $\beta 1$  and  $\beta 2$  with the recently solved crystal structure of myelin P<sub>0</sub> (Shapiro *et al.*, 1996) will allow rational choices regarding which mutations might interact with TN-R. These studies will involve the creation of stable cell lines. All cell lines, with the exception of those expressing point mutations in the extracellular regions, described below, will be cloned and characterized for mRNA and protein expression.

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### Subunit truncation mutants

Truncation mutants of  $\beta 1A$  may be constructed, sequenced, and transfected into Chinese hamster lung 1610 fibroblasts. To construct such mutants,  $\beta 1A$  cDNA is amplified by PCR. The 3' reverse primer incorporates a termination codon in place of a given residue such that the protein ends. This mutant cDNA is then subcloned into an appropriate vector such as pCMVneo (Stratagene), pcDNA3.1Zeo (Invitrogen, Carlsbad, CA) and the like. If the truncation mutants of  $\beta 1A$  and/or other  $\beta$  subunits are expressed at the plasma membrane but are not affected by TN-R in the cell migration assay, then it can be concluded that the COOH-terminal domains of these subunits are critical for transducing intracellular signals between TN-R and intracellular proteins. If these mutants behave like wild type subunits in the cell migration assay, then it can be concluded that the COOH-terminal domains are not necessary for these signal transduction events.

#### Introduction of COOH-terminal signal peptides for GPI linkage

The importance of the transmembrane domains of  $\beta$  subunits can be determined by replacing them with GPI moieties. A number of CAMs, including the TN-R receptor F3/contactin, have been shown to contain a GPI moiety which anchors the extracellular portion of the protein directly to the plasma membrane without a transmembrane domain. Nascent proteins destined to be GPI-linked have been shown to contain both  $NH_2$ - and COOH-terminal signal peptides (Udenfriend *et al.*, 1995). It is thought that the COOH-terminal signal peptide is cleaved and replaced with GPI by a putative transamidase enzyme in the endoplasmic reticulum, transamidase protein + GPI  $\rightarrow$  GPI-protein + COOH-terminal signal peptide.

The amino acid residue at which cleavage and GPI linkage occurs is termed the  $\omega$  site. An extensive literature describes the hydrophobicity requirements for the COOH-terminal signal peptide, the length of the hydrophilic spacer region, as well as the identities of amino acid residues present at the  $\omega$ ,  $\omega + 1$ , and  $\omega + 2$  sites (reviewed by Udenfriend *et al.*, 1995). cDNAs can be constructed which contain a GPI linkage in place of their transmembrane domains by creating chimeras of the extracellular  $NH_2$ -domain of each  $\beta$  subunit with the COOH-terminal signal peptide of the GPI-anchored precursor of chicken N-CAM (Cunningham *et al.*, 1987; Furukawa *et al.*, 1997).

### Chimeras

Chimeras can be generated by PCR using the following strategy: Briefly, the extracellular NH<sub>2</sub>-terminal regions of  $\beta$ 1A or any other  $\beta$  subunit is amplified by PCR from extracellular epitope-tagged  $\beta$ 1A,  $\beta$ 1 or  $\beta$ 2 plasmid templates. The PCR will incorporate antisense oligonucleotide primers which code for IVSETVIP (SEQ ID NO:10) for  $\beta$ 1 or PERDTVIP (SEQ ID NO:11) for  $\beta$ 2 (a fusion protein between the 3' end of the  $\beta$  subunit extracellular region and the 5' end of the region of N-CAM).

The COOH-terminal region of N-CAM, will also be amplified by PCR from chicken brain cDNA. The PCRs incorporate sense oligonucleotide primers that encode IVSETVIP (SEQ ID NO:10) (for use with  $\beta$ 1) or PERDTVIP (SEQ ID NO:11) (for use with  $\beta$ 2), creating a fusion protein between the COOH-terminal residues of the extracellular region of  $\beta$ 1,  $\beta$ 2 OR  $\beta$ 1A and the 5' end of the COOH-terminal region of N-CAM). The PCR products will be purified and mixed (extracellular  $\beta$ 1 + N-CAM in one reaction and extracellular  $\beta$ 2 + N-CAM in another reaction and extracellular  $\beta$ 1A + N-CAM in another reaction) for use in a second round of PCR in which the components will anneal at the common IVSETVIP (SEQ ID NO:10) region for  $\beta$ 1 + N-CAM or PERDTVIP (SEQ ID NO:11) for  $\beta$ 2 + N-CAM and be filled in by the polymerase, thus generating the desired chimeras.

The processed GPI-linked proteins expressed by transfected 1610 fibroblasts or other suitable cell line will each contain a COOH-terminal sequence that ends with TVIPA with an attached GPI lipid anchor. To determine if GPI- $\beta$ 1A, GPI- $\beta$ 1 and GPI- $\beta$ 2 are expressed at the plasma membrane as GPI-anchored proteins, transfected cells can be incubated with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme which specifically cleaves the GPI moiety and releases the protein into the cell medium. The cells are then removed by centrifugation and  $\beta$ 1,  $\beta$ 1A or  $\beta$ 2 will be immunoprecipitated from the supernatant using antibodies specific to the extracellular epitope tags and analyzed by Western blot. If GPI-linked  $\beta$ 1,  $\beta$ 1A or  $\beta$ 2 is indeed expressed in the plasma membrane of transfected cells, then these lines can be tested in the cell migration assay. If these mutants behave like cells transfected with full length  $\beta$  constructs, then it is concluded that the  $\beta$  subunit transmembrane domains are not necessary for transducing TN-R-mediated signaling events and that the  $\beta$  subunits behave similarly to F3/contactin. If these mutants are expressed but do not behave like

wild type  $\beta$  subunits in the cell migration assay, then the conclusion is that the transmembrane domains are necessary for TN-R-mediated signal transduction.

### Site-directed mutagenesis of extracellular residues

5           The crystal structure of the CAM MP  $\alpha$  has recently been reported (Smith and Goldin, 1998).  $\beta 1$ ,  $\beta 1A$  and  $\beta 2$  are structurally homologous to MP  $\alpha$  and used the alignment of MP  $\alpha$  and  $\beta 1$  to make predictions about key residues in  $\alpha\beta 1$  subunit interactions (Isom *et al.*, 1995b; McCormick *et al.*, 1998). Because  $\beta 1$ ,  $\beta 1A$ ,  $\beta 2$  and MP  $\alpha$  are structurally related CAMs, the alignment of their sequences can be used to make  
10           some predictions regarding possible interactions of the  $\beta$  subunits with TN-R. In addition, it has been shown that the third Ig loop of F3/contactin is responsible for TN-R binding (Pesheva *et al.*, 1993). This domain of F3/contactin is homologous to  $\beta 2$  (Isom *et al.*, 1995b), suggesting that the Ig loop of  $\beta 2$  may be involved in interaction with TN-R as well. The region of highest homology between F3/contactin and  $\beta 2$ , is  
15           predicted to be in the hydrophobic core of the Ig loop, based on the MP  $\alpha$  crystal structure. Mutations in this core region result in  $\beta 1$  subunits which fail to fold properly or are unstable (McCormick *et al.*, 1998). Thus, those residues are not good candidates for mutagenesis. Instead, other regions may be selected.

          Oligonucleotide-directed *In Vitro* Mutagenesis System Version 2 or Sculptor  
20           kits (Amersham Life Sciences, Arlington Heights, IL) are used to mutate selected residues, as described previously (McCormick *et al.*, 1998). Stable cells lines or transient transfections may be performed to generate cells to be tested for the mutant  $\beta$  subunits analysis.

          The mutations may be tested for plasma membrane expression by transient  
25           transfection into 1610 fibroblasts as well as immunocytochemistry using antibodies recently to  $\beta 1$ ,  $\beta 2$ , and  $\beta 1A$  subunits. Mutants that express detectable protein at the plasma membrane can then be analyzed further in the cell migration assay.

\*       \*       \*

          All of the compositions and/or methods disclosed and claimed herein can be  
30           made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may

5 be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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- U.S. Patent 5,661,025.  
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5 U.S. Patent 5,693,509.  
U.S. Patent 5,707,618.  
U.S. Patent 5,714,166.  
U.S. Patent 5,749,847.  
U.S. Patent 5,770,414.  
10 U.S. Patent 5,773,289.  
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U.S. Patent 5,789,390.  
U.S. Patent 5,795,581.  
15 U.S. Patent 5,824,544.  
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20 U.S. Patent 5,834,441.  
U.S. Patent 5,836,905.  
U.S. Patent 5,849,571.  
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## CLAIMS

- 5
1. A method of screening for a modulator of sodium channel activity comprising:
- (i) providing a cell co-expressing a sodium channel  $\beta 1A$  subunit polypeptide with a sodium channel  $\alpha$  subunit;
  - (ii) contacting said cell with a candidate modulator substance; and
  - (iii) determining the effect of said candidate substance on the sodium
- 10 channel function in said cell.
2. The method of claim 1, wherein said determining comprises comparing the sodium current density of the cell in the presence of said candidate substance with sodium current density of said cell in the absence of said candidate
- 15 substance wherein an alteration of said density is indicative of said candidate substance being a modulator.
3. The method of claim 1, wherein said candidate substance is selected from a small molecule library.
- 20
4. The method of claim 1, wherein said candidate substance alters the expression of said sodium  $\beta 1A$  subunit.
5. A method for decreasing neuropathic pain in an individual comprising
- 25 administering to said individual a modulator of a sodium channel  $\beta 1A$  subunit in an amount effective to alter the activity or level of sodium channel  $\beta 1A$  subunits of a cell in said individual.
6. The method of claim 5, wherein said modulator decreases expression of sodium
- 30 channel  $\beta 1A$  subunit in the cell of said individual.

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**FIG. 1A**

- . 19 MGTLLALVVG AVLVSAAWGG CVEVDSETEA VYGMTFKILC ISCKRRSETT  
32 AETFTETFR QKGTEEFVKI LRYENEVLQL EEDERFEGRV VWNGSRGTKD  
82 LQDLSIFITN VTYNHSGDEC HVYRLLFFDN YEHTSVVKK IHLEVVDKGG  
132 WSLVTLWQAR WRDRWKEGDR LVSHRGQLTP RSHRGKDTFF LVLETSALQH  
182 TGGQIRTPP PPTNGMCIGL HSCCVTSDGC IPISEPQACP QGPERIFCMA  
232 CCVSQAGPHW RDVGTYLRPQ WE



**FIG. 1B**

1.1 MGTLLALVVGAVLVSSAWGGCVEVDSETEAVYGMTFKILCISCKRRSETT 50

1 MGTLLALVVGAVLVSSAWGGCVEVDSETEAVYGMTFKILCISCKRRSETT 50

551 AETFTWTFRQKGTEEFVKILRYENEVLQLEEDERFEGRVVWNGSRGTKD 100  
|||||  
551 AETFTWTFRQKGTEEFVKILRYENEVLQLEEDERFEGRVVWNGSRGTKD 100

1101 LQDLSIFITNVTYNHSGD.ECHVYRLFFFDNYEHNTSVVKKIHLLEVVDKG 149  
|||||  
1101 LQDLSIFITNVTYNHSGDYECHVYRLFFFDNYEHNTSVVKKIHLLEVVDKA 150

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150 KWSLVTLWQARWR.....DRWKEGDRVLVSHR..GQLTPRSRHRGKDTPF 190
      : : : : : : : : : : : : : : : : : : : : : : : : : : : :
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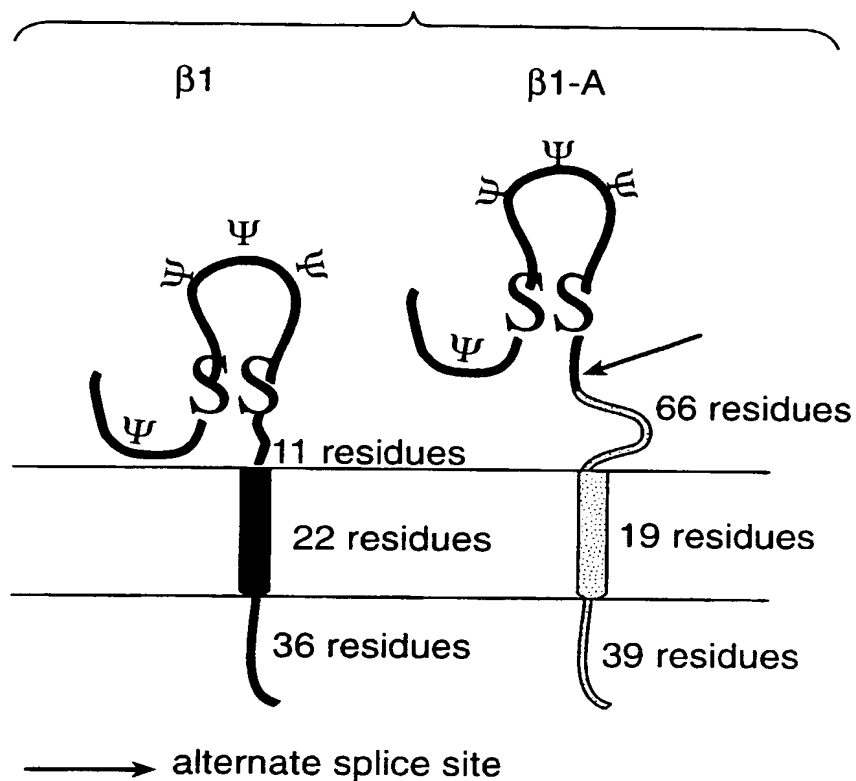
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      |      |      |      |      |
201 LAITSESKENCTGVOVAE..... 218

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240 POGPERIFCMACCVSQAGPHWRDVGTYLRPQWE..... 272

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**FIG. 1C**

**FIG. 1D**

sp|P98164|LRP2\_HUMAN LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 2  
(MEGALIN)

(GLYCOPROTEIN 330)  
Length = 1751

Score = 29.7 bits (65), Expect = 2.6  
Identities = 18/55 (32%), Positives = 22/55 (39%), Gaps = 8/55 (14%)

Query: 66 RTPTPPPT-----NGMCIGLHSCCVTSDGCIPISEPPQACPPQPERIFCMACC 112  
R PTP P NG CI + C +D C S+ C +G ER C  
Sbjct: 1219 RKPTPKPCTEYKCGNGHCIPHNDVCDADDCGDSDELGCNKGKERTCAENIC 1273

sp|Q04205|TENS\_CHICK TENSIN  
Length = 1744

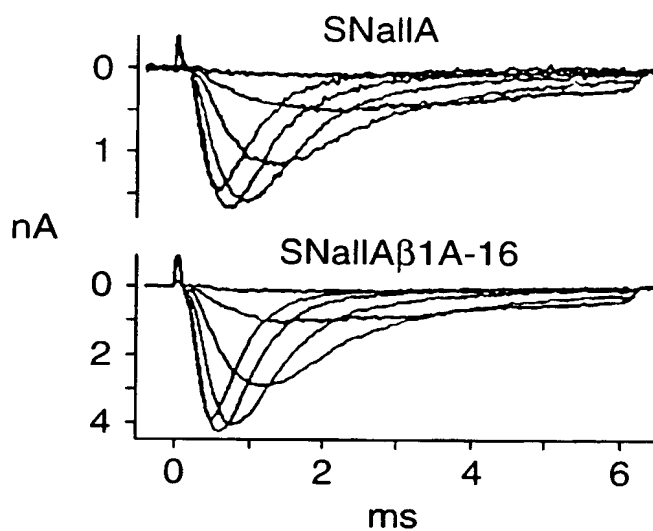
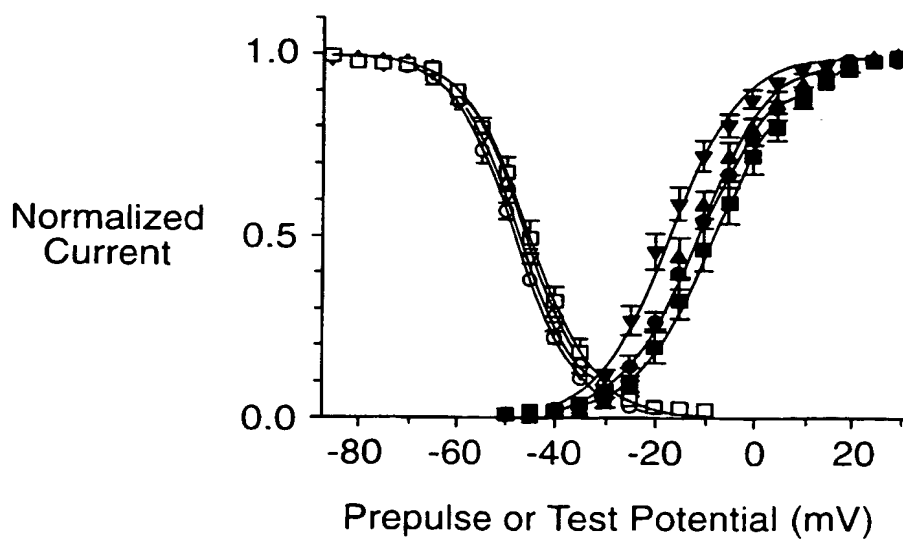
Score = 29.0 bits (63), Expect = 4.5  
Identities = 17/63 (26%), Positives = 26/63 (40%), Gaps = 6/63 (9%)

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H+ Q P + +D P +L + + Q G PTP G TS G P+  
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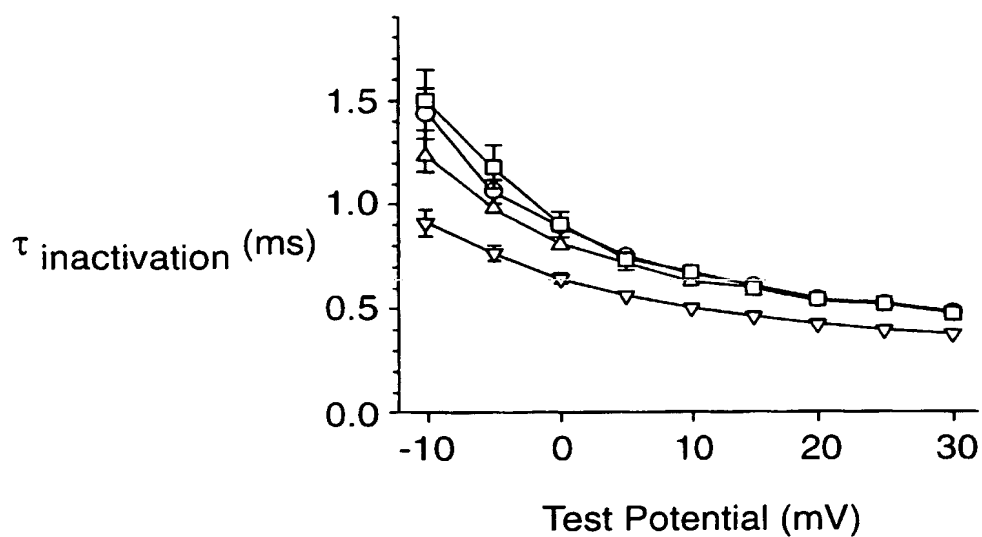
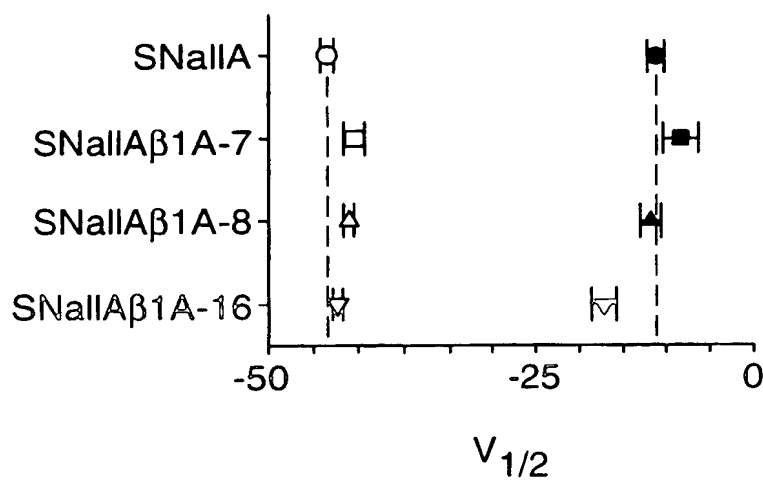
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++P

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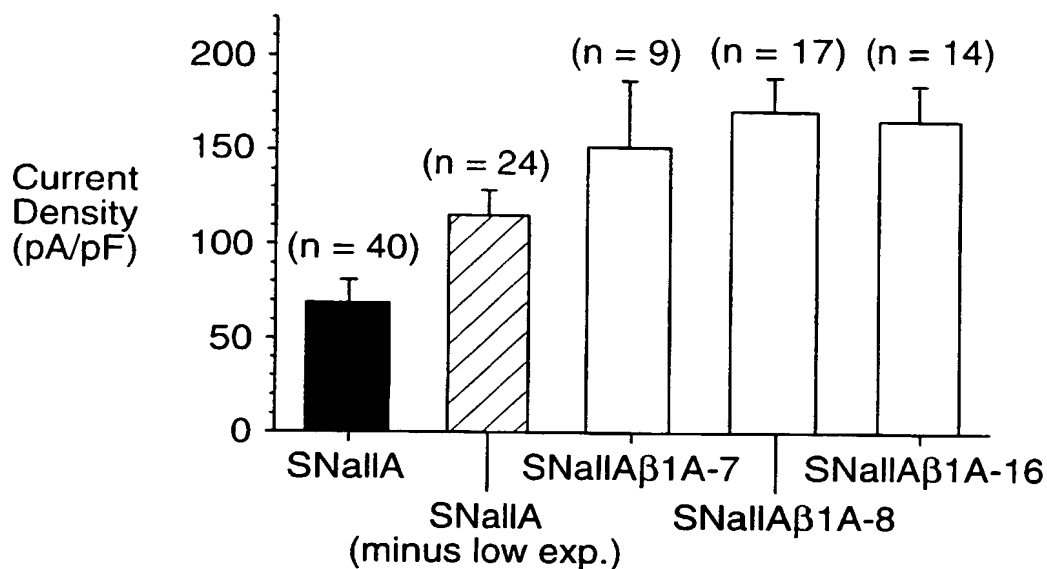
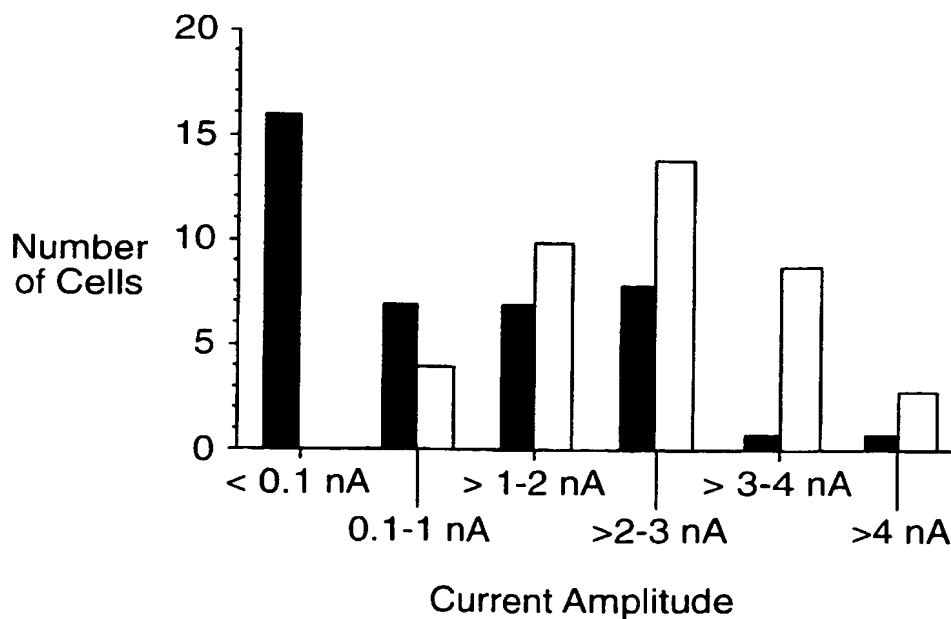
5/7

**FIG. 2A****FIG. 2B**

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**FIG. 2C****FIG. 2D**

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**FIG. 3A****FIG. 3B**

## SEQUENCE LISTING

<110> Rogers Kathryn E.  
D'Andrea, Michael

<120> Methods And Compositions Relating To Novel Sodium  
Channel  $\beta$ 1A Subunits

<130> sequence

<140> 8642/70

<141> 1999-09-30

<160> 12

<170> PatentIn Ver. 2.0

<210> 1

<211> 850

<212> DNA

&lt;213&gt; Rattus sp.

&lt;400&gt; 1

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atctcctgta agcgtcgtag tgagaccacc gccgagacct tcacggagtg gaccttcgc 180
cagaagggca cagaggaatt tgtcaagatc ctacgctatg agaatgaggt gctgcagctg 240
gaggaagatg agcgctttga gggccgtgtg gtgtggaacg gtagtcgggg caccaaggac 300
ctgcaggacc tgtccatctt catcaccaat gtcacctaca accactctgg cgactacgaa 360
tgtcacgtct accgtctcct cttctttgat aattacgagc acaacaccag cgtcgtcaag 420
aagatccacc tggaggtggt ggacaagggg aagtggagcc ttgtcactct ctggcaagcc 480
agatggaggg acagatggaa agaaggggac aggtctggtg cacacagagg ccagctaaca 540
ccccgcagcc atagggggaa ggacaccctt tttctgggtc tggagacttc agctcttcag 600
cacacaggag gtcagattag gacccccacc ccacccccca caaatggtat gtgcattgga 660
ctgcactcat gctgtgtgac ctctgacggg tgtattccta tctctgagcc ccaagcctgt 720
ccccagggac cagagagaat attctgtatg gcttgcctgt tctctcaagc gggaccccat 780
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&lt;210&gt; 2

&lt;211&gt; 272

&lt;212&gt; PRT

&lt;213&gt; Rattus sp.

&lt;400&gt; 2

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  1              5              10              15

Ala Trp Gly Gly Cys Val Glu Val Asp Ser Glu Thr Glu Ala Val Tyr
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Gly Met Thr Phe Lys Ile Leu Cys Ile Ser Cys Lys Arg Arg Ser Glu
      35              40              45

Thr Thr Ala Glu Thr Phe Thr Glu Trp Thr Phe Arg Gln Lys Gly Thr
      50              55              60

Glu Glu Phe Val Lys Ile Leu Arg Tyr Glu Asn Glu Val Leu Gln Leu
      65              70              75              80

Glu Glu Asp Glu Arg Phe Glu Gly Arg Val Val Trp Asn Gly Ser Arg
      85              90              95

Gly Thr Lys Asp Leu Gln Asp Leu Ser Ile Phe Ile Thr Asn Val Thr
      100              105              110

Tyr Asn His Ser Gly Asp Glu Cys His Val Tyr Arg Leu Leu Phe Phe
      115              120              125

Asp Asn Tyr Glu His Asn Thr Ser Val Val Lys Lys Ile His Leu Glu
      130              135              140

Val Val Asp Lys Gly Lys Trp Ser Leu Val Thr Leu Trp Gln Ala Arg
      145              150              155              160

Trp Arg Asp Arg Trp Lys Glu Gly Asp Arg Leu Val Ser His Arg Gly
      165              170              175

Gln Leu Thr Pro Arg Ser His Arg Gly Lys Asp Thr Pro Phe Leu Val
      180              185              190

```



Leu Glu Thr Ser Ala Leu Gln His Thr Gly Gly Gln Ile Arg Thr Pro  
 195 200 205  
 Thr Pro Pro Pro Thr Asn Gly Met Cys Ile Gly Leu His Ser Cys Cys  
 210 215 220  
 Val Thr Ser Asp Gly Cys Ile Pro Ile Ser Glu Pro Gln Ala Cys Pro  
 225 230 235 240  
 Gln Gly Pro Glu Arg Ile Phe Cys Met Ala Cys Cys Val Ser Gln Ala  
 245 250 255  
 Gly Pro His Trp Arg Asp Val Gly Thr Tyr Leu Arg Pro Gln Trp Glu  
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 <211> 19  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:RT-PCR  
 primercommon to B1A and B1

<400> 3  
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<210> 4  
 <211> 16  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:RT-PCR primer  
 sequence unique to B1A

<400> 4  
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<210> 5  
 <211> 4  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: B1 sequence  
 flanking intron 3

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<210> 6  
 <211> 27

<212> DNA  
 <213> Artificial Sequence

<220>  
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<400> 6  
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27

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<220>  
 <223> Description of Artificial Sequence:B1 sequence  
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<210> 8  
 <211> 27  
 <212> DNA  
 <213> Artificial Sequence

<220>  
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27

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 <213> Rattus sp.

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Gly Gln

<210> 10  
 <211> 8  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:Fusion peptide  
 to add to B1

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5

&lt;210&gt; 11

&lt;211&gt; 8

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:Fusion peptide  
sequence for B2

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1

5

&lt;210&gt; 12

&lt;211&gt; 218

&lt;212&gt; PRT

&lt;213&gt; Rattus sp.

&lt;400&gt; 12

Met Gly Thr Leu Leu Ala Leu Val Val Gly Ala Val Leu Val Ser Ser  
1 5 10 15Ala Trp Gly Gly Cys Val Glu Val Asp Ser Glu Thr Glu Ala Val Tyr  
20 25 30Gly Met Thr Phe Lys Ile Leu Cys Ile Ser Cys Lys Arg Arg Ser Glu  
35 40 45Thr Thr Ala Glu Thr Phe Thr Glu Trp Thr Phe Arg Gln Lys Gly Thr  
50 55 60Glu Glu Phe Val Lys Ile Leu Arg Tyr Glu Asn Glu Val Leu Gln Leu  
65 70 75 80Glu Glu Asp Glu Arg Phe Glu Gly Arg Val Val Trp Asn Gly Ser Arg  
85 90 95Gly Thr Lys Asp Leu Gln Asp Leu Ser Ile Phe Ile Thr Asn Val Thr  
100 105 110Tyr Asn His Ser Gly Asp Tyr Glu Cys His Val Tyr Arg Leu Leu Phe  
115 120 125Phe Asp Asn Tyr Glu His Asn Thr Ser Val Val Lys Lys Ile His Leu  
130 135 140Glu Val Val Asp Lys Ala Asn Arg Asp Met Ala Ser Ile Val Ser Glu  
145 150 155 160Ile Met Met Tyr Val Leu Ile Val Val Leu Thr Ile Trp Leu Val Ala  
165 170 175Glu Met Val Tyr Cys Tyr Lys Lys Ile Ala Ala Ala Thr Glu Ala Ala  
180 185 190

Ala Gln Glu Asn Ala Ser Glu Tyr Leu Ala Ile Thr Ser Glu Ser Lys

195

200

205

Glu Asn Cys Thr Gly Val Gln Val Ala Glu  
210 215

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
5 April 2001 (05.04.2001)

PCT

(10) International Publication Number  
**WO 01/23570 A3**

(51) International Patent Classification<sup>7</sup>: C12N 15/12,  
5/12, C07K 14/705, 16/28, G01N 33/50, 33/68, A61K  
38/17

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(21) International Application Number: PCT/US00/27034

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,  
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(22) International Filing Date:  
29 September 2000 (29.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/156,837 30 September 1999 (30.09.1999) US

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,  
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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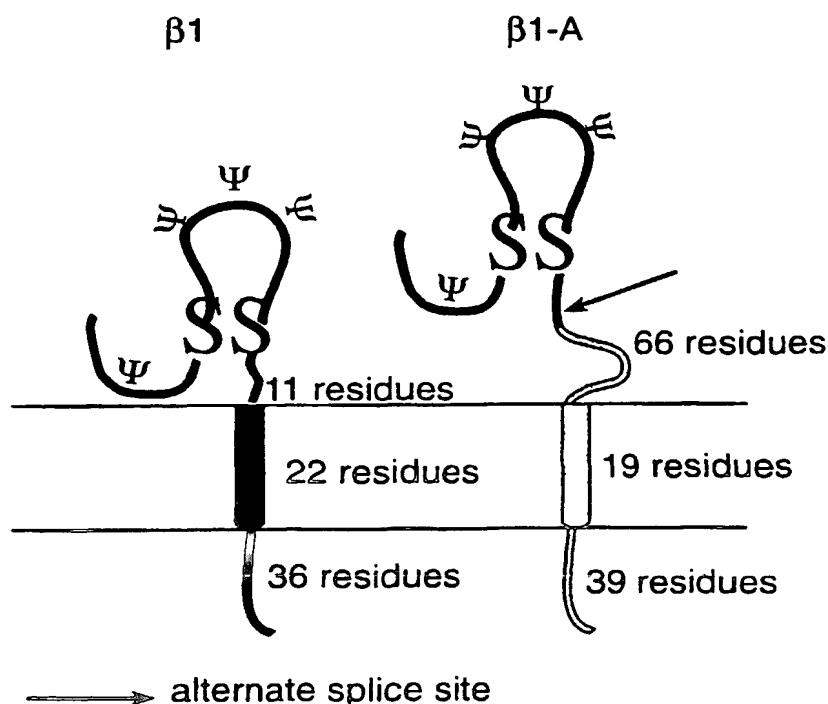
Published:  
— with international search report

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Wilmington, DE (US).

(88) Date of publication of the international search report:  
25 October 2001

[Continued on next page]

(54) Title: METHODS AND COMPOSITIONS RELATING TO SODIUM CHANNEL BETA-1A SUBUNITS



(57) Abstract: The present invention describes a novel subunit of voltage-gated sodium channels. This subunit is a splice variant of the  $\beta$  subunit of sodium channels that encodes a novel protein. Methods and compositions for using these nucleic acids and proteins of this subunit are described. The invention further discloses methods for the treatment of neuropathic pain.

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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# INTERNATIONAL SEARCH REPORT

In: International Application No

PCT/US 00/27034

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N5/12 C07K14/705 C07K16/28 G01N33/50  
G01N33/68 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, STRAND, BIOSIS, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| P, A       | KAZEN-GILLESPIE KRISTIN A ET AL:<br>"Cloning, localization, and functional<br>expression of sodium channel beta1A<br>subunits."<br>JOURNAL OF BIOLOGICAL CHEMISTRY,<br>vol. 275, no. 2,<br>14 January 2000 (2000-01-14), pages<br>1079-1088, XP002159777<br>ISSN: 0021-9258<br>the whole document<br>--- |                       |
| T          | WO 00 63367 A (UNIV CAMBRIDGE TECH ; COX<br>PETER (GB); DIXON ALISTAIR (GB); JACKSON)<br>26 October 2000 (2000-10-26)<br>the whole document<br>---<br>-/--   |                       |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- \*Z\* document member of the same patent family

Date of the actual completion of the international search

30 March 2001

Date of mailing of the international search report

05/04/2001

Name and mailing address of the ISA

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Authorized officer

Hornig, H

## INTERNATIONAL SEARCH REPORT

In. tional Application No

PCT/US 00/27034

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |  |                       |
|--|--|-----------------------|
| Category   | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
| T  | WO 00 69912 A (CURTIS RORY A J ;MILLENNIUM PHARM INC (US))<br>23 November 2000 (2000-11-23)<br>the whole document<br>---   |                       |
| A  | SUTKOWSKI E M ET AL: "BETA-1 SUBUNITS OF SODIUM CHANNELS STUDIES WITH SUBUNIT-SPECIFIC ANTIBODIES"<br>JOURNAL OF BIOLOGICAL CHEMISTRY,<br>vol. 265, no. 21, 1990, pages 12393-12399,<br>XP002159776<br>ISSN: 0021-9258<br>cited in the application<br>page 12397, left-hand column, line 12 -<br>line 42; figure 5<br>page 12398, right-hand column, line 21<br>-page 31<br>---                            |                       |
| A  | ISOM L L ET AL: "PRIMARY STRUCTURE AND FUNCTIONAL EXPRESSION OF THE BETA-1 SUBUNIT OF THE RAT BRAIN SODIUM CHANNEL"<br>SCIENCE (WASHINGTON D C),<br>vol. 256, no. 5058, 1992, pages 839-842,<br>XP002159778<br>ISSN: 0036-8075<br>cited in the application<br>the whole document<br>---  |                       |
| A  | MAKITA NAOMASA ET AL: "Voltage-gated Na <sup>+</sup> channel beta-1 subunit mRNA expressed in adult human skeletal muscle, heart, and brain is encoded by a single gene"<br>JOURNAL OF BIOLOGICAL CHEMISTRY, THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC., US,<br>vol. 269, no. 10, 1994, pages 7571-7578,<br>XP002145888<br>ISSN: 0021-9258<br>cited in the application<br>the whole document<br>--- |                       |
| A  | MARBAN E ET AL: "STRUCTURE AND FUNCTION OF VOLTAGE-GATED SODIUM CHANNELS"<br>JOURNAL OF PHYSIOLOGY, XX, XX,<br>vol. 508, no. 3, 1 May 1998 (1998-05-01),<br>pages 647-657, XP000938782<br>ISSN: 0022-3751<br>the whole document<br>---<br>-/--   |                       |



# INTERNATIONAL SEARCH REPORT

Int .tional Application No

PCT/US 00/27034

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|----------|--|-----------------------|
| A        | <p>BELCHER S M ET AL: "Cloning of the cDNA encoding the sodium channel betal subunit from rabbit"<br/>           GENE,NL,ELSEVIER BIOMEDICAL PRESS.<br/>           AMSTERDAM,<br/>           vol. 170, no. 2, 8 May 1996 (1996-05-08),<br/>           pages 285-286, XP004042842<br/>           ISSN: 0378-1119<br/>           the whole document<br/>           -----</p> |                       |

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 5,6

Claims 5 and 6 refer to a method for decreasing neuropathic pain in an individual comprising administering to said individual a modulator of a sodium channel beta1A subunit without giving a true technical characterization of the modulator. Moreover, no such compounds are defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art.5 and 8 PCT). No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/27034

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| WO 0063367 A                              | 26-10-2000          | AU 3285100 A               | 02-11-2000          |
| WO 0069912 A                              | 23-11-2000          | AU 5010900 A               | 05-12-2000          |

Form PCT/SA/210 (patent family annex) (July 1992)

